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Some Laboratory Data on Hedgehogs, Hibernating and Non-hibernating.

By

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The present interest in hypothermia for medical purposes necessitates a better understanding of the metabolic processes at low temperatures. Knowledge of the mechanisms by means of which life is preserved in hibernators would be of particular interest. Although very thorough surveys have been made in this fascinating field (BENEDICT and LEE 1938, JUVENELLE et al. 1952, KAYSER 1953, SUOMALAINEN 1953, LYMAN and CHATFIELD 1955), there is evidently need for collecting more data on basic biochemical relationships. We have chosen to study the blood (blood corpuscles, blood sugar, plasma proteins and lipids, protein bound iodine and some inorganic ions), the urine (sugar, ketone bodies and albumin) and some tissues (cytochrome c, calcium and magnesium) in hibernating and non-hibernating hedgehogs.

Material and Methods.

Hedgehogs (Erinaceus europaeus), hibernating and non-hibernating, were decapitated and the mixture of arterial and venous blood from the neck vessels was used for analysis of the blood. Urine was collected by puncture of the bladder.

Skin temperatures were measured by means of a thermocouple, as described earlier (BIÖRCK and JOHANSSON 1955).

Hemoglobin was determined photometrically. Determination of red and white blood corpuscles as well as differential counts were performed

with conventional hospital laboratory methods. For thrombocytes, KRISTENSON's method (ASTRA 1947) was used, and for total eosinophils the method of BERGSTRAND, HELLSTRÖM and JOHANSSON (1950). The reticulocytes were counted after treatment with methylene blue (ASTRA 1947). Hematocrits were read after centrifugation for 60 minutes at 3,000 revolutions/min. The fractionation of plasma proteins and lipids was made by means of paper electrophoresis acc. to LAURELL, LAURELL and SKOOG (1956). For barbiturates, the spectrophotometric method of LOUS (1954) was employed. Protein-bound iodine was determined acc. to SKANSE and HEDENSKOG's modification of the method of BARKER, HUMPHREY and SOLEY (1951). For urine analysis conventional hospital laboratory methods were employed. The above determinations were carried out in the chemical laboratory of Allmänna Sjukhuset during 1955. Sodium and potassium were determined by means of flame photometry in non-hibernating hedgehogs in 1955 and in hibernating ones in 1955 and 1956. Prior to these studies, some analyses were carried out in 1954 at the research laboratory of the Leo Co., Hälsingborg[1], on blood sugar as well as calcium and magnesium in heart, brain, skeletal bones, carcass and blood serum and cytochrome c in the heart. (Preliminary studies had shown that cytochrome c values in other organs could not be estimated with any accuracy). The content of calcium, magnesium and cytochrome c of the organs was determined and expressed as mg per gram of dry weight. Dry weight was determined after 16 hours at 105° C. Blood sugar was determined according to HAGEDORN-JENSEN. Calcium and magnesium were determined by application of micromethods. The total of calcium and magnesium was determined acc. to SOBEL and HANOK (1951). Calcium was titrated photometrically acc. to FALES (1953) and magnesium calculated as the difference between these two values. Cytochrome c was determined by means of a modification of a method worked out by LOFTFIELD and BONNICHSEN on the basis of earlier works by PALÉUS and NEILANDS (1950).

The animals in the 1954 study are designated series A, those of the 1955 study series B and those of the 1956 study series B_1 in the tables. In series A the hibernating animals were killed in late February to early March, and the non-hibernating in September. In series B, 8 hibernating animals were killed late in January and 5 in the middle of March, whereas the non-hibernating ones were killed in the latter part of June. In series B_1, 7 hibernating animals were killed in the beginning of February. All the animals were kept in separate cages and received, in periods, when they were non-hibernating, kitchen garbage and water. During hibernation, they all had access to water but not to food.

Results.

Data on weight and skin temperature are given in table 1. Skin temperatures in series A were not recorded in detail, but there was

[1] These analyses were carried out by S. VEIGE.

Table 1.

		Number of animals	Weight in gram		Temperature in ° C.	
			Mean	Range	Mean	Range
Series A	Non-hibernating .	9	788	500—1.010		
	Hibernating	9	644	540— 790		
Series B	Non-hibernating .	15	807	600— 950	33.°7	32.°5—34.°5
	Hibernating	13	546	250— 800	7.°8	4.°0 -11.°0
Series B_1	Hibernating	7	530	350— 750	7.°4	5.°0—12.°0

no doubt as to the hibernating state. The laboratory data obtained appear from the tables 2 to 6, where the number of observations, the mean value, and the range are given.

Discussion.

Blood corpuscles.

Earlier data on the number of *red blood corpuscles* and the *hemoglobin* levels in hibernators during the different seasons are somewhat confusing. Most authors maintain that there is a decrease at the end of the hibernation period (QUINCKE 1882, DUBOIS 1896, POLIMANTI 1912), while RASMUSSEN (1916) was unable to confirm this. STUCKEY and COCO (1942) and SUOMALAINEN (1954) have reported a decrease during hibernation, in contrast to SVIHLA, BOWMAN and RITENOUR (1953) who found an increase. However, details are lacking as to which part of the hibernation period that was studied. Our results from the end of the hibernation period (table 2) showed an obvious decrease of red blood corpuscles and hemoglobin in comparison to the January values, while the latter showed no definite difference from the summer values. This finding is in conformity with the majority of previous observations also on other hibernators (woodchucks, ground squirrels) and may explain why values from only the early hibernation period can given an erroneous impression.

It is also worth noting that the *hematocrit* values (table 3) were considerably lower in March — 35 volumes per cent — than in January — 58 volumes per cent. The value from non-hibernating animals was 45 volumes per cent. These findings are in agreement with those of MCBIRNIE, PEARSON, TRUSLER, KARACHI and BIGELOW (1953) and SVIHLA et al. (1953), who also found higher

Table 2.

	Hemoglobin (%)			Red corpuscles (millions/mm³)			White corpuscles (number/mm³)		
	Number of animals	Mean	Range	Number of animals	Mean	Range	Number of animals	Mean	Range
Non-hibernating	11	100	80—121	11	8.96	6.90—10.80	13	12,560	4,200—23 800
Hibernating in Jan.	8	94	84—110	8	9.28	8.40—10.20	8	2,325	800— 4300
Hibernating in March	5	74	66— 80	5	7.43	7.10— 7.80	5	1,620	900— 2300

	Number of animals	Differential count (per 100 counted cells)									
		Neutrophils (%)		Eosinophils (%)		Basophils (%)		Lymphocytes (%)		Monocytes (%)	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Non-hibernating	13	57	46—71	5	0—10	0.3	0—2	36	23—46	2	0—5
Hibernating in Jan.	8	59	45—71	0.4	0—2	0.1	0—1	38	26—51	1.8	1—3
Hibernating in March	5	66	60—75	0.2	0—1	0.2	0—1	33	24—38	1.2	1—2

	Blood platelets (number/mm³)			Reticulocytes (per 1,000 cells)			Eosinophils (number/mm³)		
	Number of animals	Mean	Range	Number of animals	Mean	Range	Number of animals	Mean	Range
Non-hibernating	8	432,000	241,000— 880.000	12	54	15—176	12	993	138—2,388
Hibernating in Jan.	7	875,000	402,000—1,170,000	6	12	4—20	6	15	0—31
Hibernating in March	1	796,000		5	11	0—25	5	10	0—38

values during one period of the hibernation than in their non-hibernating animals. It would be of considerable interest to determine blood volume in hibernators during various phases of the hibernating periods, in order to establish, whether the apparent reduction in corpuscular elements is to some extent caused by a dilution of the blood plasma, secondary to the overall metabolic changes during the utilization of the nutritional stores.

Pertitent in this respect may be a few observations on microscopic sections of the *spleen*. During hibernation, there is a considerable increase in the size of this organ. The summer spleen was found to be rather poor with regard to sinusoids and, furthermore, to contain a "normal" number of erythrocytes and a considerable amount of hemosiderin. Spleens from hibernating animals were found to show a maximal dilatation of the sinusoids, disrupting the normal pattern of the lymphoid tissue, many megakaryocytes and a great deal of hemosiderin depots, but no erythrocytes were observed in the sinusoids, giving an impression of a block between the splenic and the systemic circulation. The significance of this finding is still uncertain.

It is generally agreed that the number of *leucocytes* decreases in hibernation[1] (QUINCKE 1882, CARLIER 1893, DUBOIS 1896, ARGAUD and BILLARD 1911, POLIMANTI 1912, RASMUSSEN 1916, SVIHLA et al. 1953, SUOMALAINEN 1953). Our findings are in agreement with theirs (table 2).

Several hypotheses have been proposed regarding the cause of this decrease. BRACE (1952) stated that the erythropoësis in hibernating woodchucks was arrested and that the same was true of the lymfopoësis in the spleen. In our hedgehogs, the reticulocytes were considerably lower during hibernation (table 2) than when the animals were non-hibernating. The most likely explanation, therefore, would be a general slowing down of the formation of the various blood corpuscles. If the maturing and destruction of these elements is proportionately retarded, the net effect would be status quo. As this apparently is not the case, the formation arrest is probably more severe than the effects on survival and destruction (provided there is no increase in blood volume during hibernation). A few microscopic studies of the *bone marrow* showed an apparently greater number of blood-forming cells during hibernation than in summer animals, but do not permit any conclusions in this respect.

[1] The same seems to be true also in induced hypothermia in many non-hibernators. Villalobos et al. (1955) observed the number of leucocytes to decrease by 80 % in dogs at a body temperature of 17° C.

Table

		Blood sugar (g %)		
		Number of animals	Mean	Range
Series B	Non-hibernating in June			
	Hibernating in January			
	Hibernating in March			
Series A	Non-hibernating in September ..	9	0.144	0.121—0.179
	Hibernating in February—March	7	0.073	0.060—0.087

ARGAUD and BILLARD (1911) found only mononuclear leucocytes in dormice, who "hibernated" during the summer by inanition. RASMUSSEN (1916) reported only slight changes in *the differential leucocyte count* in woodchucks; possibly the mononuclear elements decreased more than other forms. STUCKEY and COCO (1942), on the contrary, reported an increase in monocytes and lymphocytes but a decrease of neutrophil leucocytes in hibernating ground squirrels. SUOMALAINEN again (1953) found the opposite to be true of hedgehogs. Our results indicate only minor changes in these elements and are thus in agreement with RASMUSSEN's. Hibernation is accompanied by a drastic eosinopenia, however; in our series there is a drop from a total eosinophil count in summer hedgehogs of 993/mm³ to 15/mm³ in winter hedgehogs. SUOMALAINEN also reports a reduction from 4 per cent to nil at standard differential counts in hibernating hedgehogs. As regards non-hibernators views are conflicting: in most cooled dogs VILLALOBOS, ADELSON and BARILA (1953) found no significant trend in blood smears, while HELMSWORTH, STILES and ELSTUN (1955) registered a reduction of the total eosinophil count by 86 per cent.

During hibernation, the circulation of blood seems to be considerably slowed down. This would facilitate intravascular clotting. Several authors report a prolonged clotting time during hibernation as a mechanism to avoid this complication. SUOMALAINEN (1953) found an increase in the number of heparinocytes (Mastzellen) in hibernating hedgehogs, while SVIHLA et al. (1953) found a reduction in the number of *blood platelets* in ground squirrels. (This was also found by VILLALOBOS et al. (1953) and HELMSWORTH et al. (1955) in hypothermic dogs.) In our series, the number of blood platelets was increased during hibernation, an unexpected finding, which is hard to explain. Studies

3.

Protein-bound iodine (γ %)			Hematocrit (%)		
Number of animals	Mean	Range	Number of animals	Mean	Range
3	3.4	2.8—3.8	6	45	40—55
4	2.0	1.6—2.4	2	58	55—61
1	1.7		3	35	32—37

in progress presently seem to indicate some differences in the content of humoral coagulation factors. It is, furthermore, conceivable that the temperature effect on the various enzymatic reactions that ultimately cause the coagulation of blood must be very important.

Blood chemistry.

It is well known, that *blood sugar* decreases in hibernation (LYMAN and CHATFIELD 1955); our results agree with this (table 3). The only exceptions to this rule seems to be hamsters (LYMAN and LEDUC 1953) and arctic ground squirrels (MUSACCHIA and WILBER 1952); this may at least in the hamsters be due to the habit of these animals to interrupt their hibernation now and then for a meal.

Observations by CUSHING and GOETSCH (1915) and ADLER (1926) indicate an involution of the *thyroid* during hibernation. We have been able to show that protein-bound iodine, as an expression of circulating thyroid hormone, is lower in hibernating than in non-hibernating animals (table 3). This is in conformance with LACHIVER's (1952) values for the marmot. The summer values are of the same order as in other animals (KATSH and WINDSOR 1955). The earlier theory of ADLER (1926) that low thyroid activity induces hibernation is generally not accepted; the decrease in activity of the thyroid, is probably a result and not a cause of hibernation. On the other hand, it may be instrumental in maintaining the metabolic depression in hibernation.

Attempts were also made to test a suggestion that endogenous production of *barbiturate* might be an explanation of hibernation. Barbiturate, however, was neither found in the serum from two hibernating hedgehogs in the middle of the winter, nor in that

Table 4.

Protein electrophoreses.

	Hibernating in January			Hibernating in March			Non-hibernating in June		
	Number of animals	Mean	Range	Number of animals	Mean	Range	Number of animals	Mean	Range
Total protein (g %)	6	7.7	7.2 —8.2	3	8.0	7.9 —8.2	15	8.0	7.4 —8.5
Albumin (g %)	6	4.09	3.46—4.34	3	4.00	3.48—4.62	15	3.13	2.02—4.52
α_1-glob. (g %)	6	0.65	0.53—0.94	3	0.71	0.67—0.79	15	0.60	0.49—0.92
α_2-glob. (g %)	6	0.36	0.29—0.47	3	0.43	0.36—0.57	15	0.46	0.35—0.79
β_1-glob. (g %)	6	0.93	0.83—1.06	3	1.18	0.86—1.46	15	1.26	0.90—1.71
β_2-glob. (g %)	6	1.17	0.99—1.42	3	1.22	1.12—1.42	15	1.66	0.79—2.29
γ-glob. (g %)	6	0.52	0.42—0.63	3	0.48	0.25—0.72	15	0.90	0.46—1.36

Lipid electrophoreses.

	Hibernating in January			Hibernating in March			Non-hibernating in June		
	Number of animals	Mean	Range	Number of animals	Mean	Range	Number of animals	Mean	Range
Total lipids (mg %)	8	875	650—1,170	5	802	704—973	15	732	476—1245
α (%)	8	62	46—75	5	76.5	58.7—89.3	15	65.5	44.0—83.5
β (%)	8	30	20—41	5	17.3	6.6—36.2	15	22.4	11.5—37.2
γ (%)	8	8	2—20	5	6.2	4.1—9.0	15	12.1	0.0—25.6

Table 5.

		Sodium (m Eq/L)			Potassium (m Eq/L)		
		Number of animals	Mean	Range	Number of animals	Mean	Range
	Non-hibernating in June	11	155	146—167	9	6.6	5.9—7.7
Series B	Hibernating in January	3	168	161—180	2	7.0	6.8—7.1
	Hibernating in March	4	144	116—185	4	7.1	6.4—8.0
Series B_1	Hibernating in February	7	155	150—162	5	6.0	5.7—7.1

		Calcium (m Eq/L)			Magnesium (m Eq/L)		
		Number of animals	Mean	Range	Number of animals	Mean	Range
Series A	Non-hibernating in September	7	3.75	2.30—4.85	7	3.85	2.71—5.25
	Hibernating in February—March	8	4.35	3.20—5.15	8	3.20	1.64—3.85

from two hedgehogs in the month of November, of which one had just started to hibernate while the other was still non-hibernating. This is a time, when the highest values would be expected.

The values of our *protein and lipid electrophoreses* are given in table 4. Because of the increase in hematocrit in early hibernation one would expect the electrophoretical values to be higher when the animals are hibernating provided there is no endogenous disturbance in the protein and lipid metabolism. In table 4 it is seen that albumin follows this pattern in January as compared to June but this is neither the case with the β_1- and β_2-fraction nor with the γ-fraction. In the other fractions, including the lipids, no consistent changes were found. More data are needed, but our findings indicate differences in behaviour of the albumin and globulin fractions, the β-fractions and especially the γ-fraction decreasing in hibernation. In the hibernating hedgehog, the balance between production and destruction of the more high-molecular proteins seems to be maintained with some difficulty. While this may signify a lower immunological defence level, it may be caused by the absence of adequate stimuli for continuous antibody formation. — Recent investigations on patients exposed to artificial cooling (24° C—28° C) as a preparation for operation (WULFF 1956) show only a slight decrease of the total protein and of all the fractions proportionately.

With regard to determinations of *plasma electrolytes*, differences of opinion are even more frequent in the literature on hibernation and hypothermia than as regards corpuscular elements. Recent reviews on this subject are those of KAYSER (1953) for hibernators and HÆGER (1954) for induced hypothermia in non-hibernators. SUOMALAINEN (1953) found an increase in sodium and a surprising decrease in potassium concentration during hibernation in hedgehogs, while MCBIRNIE et al. (1953) found an increase in potassium in hibernating groundhogs. RIEDESEL and FOLK (1956) found no significant change in bats. Our data (table 5) show small changes. The potassium concentration was unaltered.

Likewise, the differences observed by us for calcium and magnesium are not statistically significant. MCBIRNIE et al. (1953) and RIEDESEL and FOLK (1956) reported an increase of magnesium during hibernation and SUOMALAINEN (1939) found an increase also of calcium. Also in artificial cooling of homoiothermic and non-homoiothermic animals, an increase in magnesium has been reported (PLATNER 1950). — We were interested to see if any movements of magnesium out of specific tissues to the plasma could be detected (table 6). The proportions between plasma magnesium

Table 6.

	Hibernating in February—March			Non-hibernating in September		
	Number of animals	Mean	Range	Number of animals	Mean	Range
Heart:						
Ca mg/g dry weight	9	0.32	0.21—0.41	8	0.31	0.22—0.41
Mg mg/g dry weight	9	0.82	0.75—0.90	8	1.15	1.00—1.43
Cytochrome c mg/g dry weight	8	0.82	0.69—1.03	8	0.92	0.77—1.07
Brain:						
Ca mg/g dry weight	9	1.18	0.28—4.1	9	1.09	0.21—3.84
Mg mg/g dry weight	9	0.84	0.65—1.30	9	0.83	0.62—1.20
Skeletal bone:						
Ca mg/g dry weight	7	189	159—210	9	193	174—214
Mg mg/g dry weight	7	10.6	1.5—22.5	9	8.5	4.1—13.1
Carcass:						
Ca mg/g dry weight	8	33.2	26.2—43.9	9	36.6	24.0—52.3
Mg mg/g dry weight	8	4.2	3.16—5.03	9	1.5	< 1—3

and the magnesium stores in the tissues, however, together with the errors of the method whenever the calcium values greatly exceeded the magnesium values, made this attempt unsuccessful; the values in table 6 are given only for general information. The heart, however, is an exception, as all winter values were considerably below the summer values. In view of the great importance of magnesium for the ionic milieu in muscular contraction, this observation will be subject to further investigation.

According to TISSIÈRES (1946) and DRABKIN (1948), the content of *cytochrome c* in muscles is related to the metabolic rate and thyroid activity. It was, therefore, of interest to investigate whether there were any changes in the cytochrome c content of the heart in hibernation. No significant reduction was observed, however. The amount of heart muscle cytochrome c in both summer and winter hedgehogs is of the expected magnitude for an animal of this size and activity (BIÖRCK, 1956). Despite low thyroid function, these animals apparently manage to conserve a normal content of respiratory enzymes, which is a prerequisite for immediate activity whenever arousal is prompted.

Urine analysis.

Urine sugar, acetone or aceto-acetic acid were found neither in hibernating hedgehogs nor in non-hibernating ones. Instead albumin was found in half of the non-hibernating animals and in all of the hibernating ones.

This is probably a real albuminuria for in hibernating animals the Heller reaction was still positive when the urine had been centrifugated with 3,000 revolutions/min. for 10 min. The sediments produced in this way showed 2 to 4 red corpuscles and 5 to 10 white per visual field. The specific gravity was high in the hibernating animals (1,033). It is worth noting that rabbits kept at a body temperature of 28° C for about two days also showed albuminuria. There was also glycosuria, but neither acetone nor aceto-acetic acid.

Summary.

Comparative biochemical studies have been performed in hibernating hedgehogs in January and March and in non-hibernating hedgehogs in June.

Red blood corpuscles and hemoglobin level were lower in March in comparison to January and June when the difference was slight.

Also the hematocrit values were lower in March compared with January, while the values in June lay between these. The leucocytes decreased during hibernation but the differential leucocyte count did not change markedly except for the eosinophils which decreased comparatively more than the other leucocytes. Also reticulocytes decreased but blood platelets increased.

The blood sugar decreased as did the protein-bound iodine. — No significant changes in serum potassium or sodium were found.

At electrophoresis an increase of the albumin fraction was found, while β_1-, β_2- and γ-globulins decreased.

From the tissue studies — made on hibernating hedgehogs in late February to early March and on non-hibernating ones in September — it can be seen that the magnesium content of the heart was lower during winter; the cytochrome c content, however, did not change.

Sugar, aceton or aceto-acetic acid were not found in the urine. Urinary albumin, however, appeared in all the hibernating animals and in half of the non-hibernating ones.

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Experiments on the Potassium Absorption of the Erythrocytes of Rana Esculenta and Rana Temporaria after Bleeding and in Hypertonic Plasma.[1]

By

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In a former paper (ØRSKOV 1948) it was shown that bacillus coli communis in a hypertonic sodium chloride solution absorbs potassium after which the plasmolysis will disappear. This process is very fast, it is finished in a few minutes, and during this time the cell potassium may have increased by 30 to 70 %. Similar observations were made on red blood cells from pigeon and hen (ØRSKOV 1954), and the present paper concerns blood from frogs.

Methods.

Determination of potassium, sodium and haematocrit-values were made as previously described (ØRSKOV 1952 and 1954).

The frogs were kept at 12° and in darkness. One hour before the bleeding 0.2 ml heparin (650 units per ml) were injected into the dorsal lymph sac. The vertebral column was sectioned just below the head, and the blood collected in centrifuge glasses. In the experiments where it was desired that the spontaneous potassium absorption should be avoided, the frogs were kept for one hour in ice-water before the bleeding (temperature of the water about 2°). The blood is divided into two equal parts to one of which is added 1/20 volume 0.7 % NaCl to the

[1] Partly published im Abstracts Of Communications, 20. International Physiological Congress, Brussels 1956, page 694.

other 1/20 volume of a hypertonic NaCl solution. After varying times at 22° the blood is examined. The cooling probably prevents the liberation of adrenaline and noradrenaline into the blood.

Results.

As is the case with pigeon blood the frog red blood cells absorb potassium from plasma spontaneously after bleeding, and this process can be surprisingly rapid. The rapidity is most pronounced when the time between capture and bleeding is short. In 6 experiments with ranae esculentae in the first part of October the red blood cells increased their potassium concentration with about 6 mM/l in one hour at 20°, and the potassium concentration of the plasma decreased from about 4 mM/l to below 1 mM/l. Likewise it can be shown that the red blood cells react with a faster potassium absorption after hypertonic sodium chloride addition early in the autumn.

The reason for the spontaneous potassium absorption will be discussed in a following paper. To save space the above-mentioned experiments are not stated in tables.

Most of the experiments have been carried out with ranae esculentae. To save space only the experiments with ranae temporariae shall be stated as the results are quite similar. The experiments in table I were made in December 1955. In all experiments addition of hypertonic sodium chloride solution provokes a potassium absorption. The rise of the osmotic pressure is about 10 and 25 %, and the final increase of potassium of the cells corresponds to about 40 % of the rise in osmotic pressure.

Summary.

It is shown that the erythrocytes of ranae esculentae and temporariae behave very much as those from pigeons.

The blood taken from the neck of the frogs shows a marked, and in some cases a very rapid absorption of potassium from the plasma. If the frogs are cooled (2°) for an hour before the bleeding the erythrocytes show no spontaneous absorption.

Addition of hypertonic sodium chloride solution is followed by a potassium absorption of the red blood cells.

Table I.

Ranae Temporariae. All frogs cooled in icewater for one hour.

a = added 1/20 volume 0.7 % NaCl
b = added 1/20 volume 350 mM/l NaCl
c = added 1/20 volume 700 mM/l NaCl

Time at 22°			Plasma Na mM/l	Plasma K mM/l	Haematocrit-values	Increase of Cell K mM/l	Average
1 hour	1.	a	104	2.87	27		
		b	118	2.54	25	0.8	
	2.	a	110	3.74	22		
		b	122	2.97	21	2.6	1.4
	3.	a	100	3.84	33		
		b	110	3.00	32	0.9	
2 hours	1.	a	107	3.35	27		
		b	122	2.78	26	1.4	
	2.	a	103	4.04	27		2.2
		b	116	3.25	26	2.0	
	3.	a	110	3.37	13		
		b	125	2.89	12	3.2	
4 hours	1.	a	106	6.27	23		
		b	114	5.37	22	2.8	
	2.	a	106	3.55	20		3.4
		b	120	2.93	20	2.5	
	3.	a	105	5.19	22		
		b	118	3.82	22	4.9	
18 hours	1.	a	113	2.67	22		
		b	127	1.61	22	3.7	
	2.	a	108	4.13	26		3.7
		b	124	2.60	25	4.3	
	3.	a	105	4.89	23		
		b	119	3.88	22	3.2	
1 hour	1.	a	103	3.27	19		
		c	135	2.69	18	2.4	
	2.	a	106	3.45	28		1.6
		c	135	2.93	26	1.1	
	3.	a	108	3.32	25		
		c	138	2.81	23	1.3	
2 hours	1.	a	106	3.72	21		
		c	130	2.62	20	4.4	
	2.	a	102	3.19	27		4.1
		c	139	2.02	26	3.1	
	3.	a	110	4.08	31		
		c	140	1.85	28	4.8	
4 hours	1.	a	105	4.11	22		
		c	140	1.88	22	7.9	
	2.	a	109	3.25	13		8.2
		c	135	2.00	13	8.4	
	3.	a	106	3.90	26		
		c	135	1.75	25	8.3	
18 hours	1.	a	104	3.24	18		
		c	139	0.66	17	11.7	
	2.	a	107	3.60	23		9.9
		c	142	1.09	22	8.4	
	3.	a	107	3.48	21		
		c	140	0.89	20	9.7	

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Experiments on the Influence of Adrenaline and Noradrenaline on the Potassium Absorption of Red Blood Cells from Pigeons and Frogs.[1]

By

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In preceding papers it has been demonstrated that blood from pigeons and frogs shows a spontaneous potassium absorption from the plasma. If the frogs have been cooled for one hour in ice-water the blood will show no spontaneous potassium absorption. It is probable that the cooling of the frogs prevents a liberation of adrenaline and noradrenaline into the blood. In any case it can be demonstrated that adrenaline and noradrenaline when added to the blood will cause a potassium absorption, and that this effect can be shown at very low concentrations.

The methods employed are the same as in the preceding paper (ØRSKOV 1956) except that the hypertonic solution is exchanged for a solution of adrenaline or noradrenaline in 0.7 % NaCl.

As in experiments with hypertonic sodium chloride the blood cells show the greatest potassium absorption if the frogs are used a short time after they have been caught.

In table I, where high adrenaline concentrations were used, an increase of cell potassium of from 5 to 15 % is seen after 18 hours at 22° (normal cell potassium — average 110 mM/l).

In table II adrenaline and noradrenaline in varying concentrations are added. There is probably an effect of 0.01 μg/ml of adrenaline, and for noradrenaline between 0.1 and 1.0 μg/ml. By increasing concentrations the effect increases remarkably

[1] Partly published in Abstracts Of Communications, 20. International Physiological Congress, Brussels 1956, page 694.

Table I.

The haematocrit values for ranae esculentae ranged between 12 and 22, and for ranae temporariae between 18 and 29.

a = added $^1/_{50}$ volume 0.7 % NaCl
b = added $^1/_{50}$ volume 0.7 % NaCl + adrenaline
Final concentrations in the blood 10 μg/ml except in the three first experiments where they were 20, 5 and 1 μg/ml respectively.

		Ranae Esculentae		*Ranae Temporariae*	
		Plasma K[1] mM/l	Increase of Cell K mM/l	Plasma K mM/l	Increase of Cell K mM/l
1 hour at 22°	1	a 4.05 b 3.20	3.09	a 4.09 b 3.79	0.8
	2	a 4.14 b 3.66	3.5	a 3.79 b 3.73	0.2
	3	a 3.55 b 3.21	2.0	a 4.37 b 4.11	0.7
2 hours at 22°	4	a 4.40 b 3.28	6.3	a 3.78 b 3.34	1.5
	5	a 4.50 b 4.24	1.2	a 3.38 b 3.12	1.0
	6	a 4.78 b 4.26	3.2	a 3.63 b 3.25	1.3
4 hours at 22°	7	a 6.87 b 4.89	7.1	a 3.89 b 3.40	2.2
	8	a 4.81 b 3.13	10.7	a 3.71 b 2.46	4.7
	9	a 4.28 b 3.86	3.8	a 3.89 b 2.71	4.7
18 hours at 22°	10	a 6.32 b 1.67	18.6	a 4.12 b 2.08	6.1
	11	a 4.25 b 2.46	11.0	a 4.85 b 1.10	9.7
	12	a 6.75 b 4.00	12.5	a 5.15 b 3.87	4.3

[1] final values.

slowly. In the last experiments of the table high concentrations of adrenaline or noradrenaline and hypertonic plasma are used at the same time, and the effect is very marked, which is seen from the increase of cell potassium, but best from the extremely low potassium concentrations of the plasma at the end of the experiments.

Experiments with Blood from Pigeons.

Simultaneously with the above-mentioned experiments a method was found to demonstrate the effect of adrenaline and noradrenaline on the plasma potassium of pigeons. In many control experiments where the pigeon blood is rocked in Erlenmeier

Table II.

Ranae Temporariae.

Hours at 22°	Concentration of Adrenaline or Noradrenaline in Blood	Final Plasma K mM/l	Increase of Cell K mM/l	Average
4		4.4	0.9	
18	0.01 μg/ml adrenaline	3.3	0.9	0.9
18		4.4	0.9	
4		4.3	0.7	
18	0.01 μg/ml noradrenaline	5.1	— 0.5	0.1
18		5.3	0.0	
4		3.5	1.3	
18	0.1 μg/ml adrenaline	3.3	3.8	2.5
18		4.0	2.4	
4		5.5	0.1	
18	0.1 μg/ml noradrenaline	5.1	0.0	0.2
18		3.9	0.5	
4		4.1	1.9	
18	1 μg/ml adrenaline	3.0	6.9	4.4
18		3.9	4.4	
4		4.3	1.0	
18	1 μg/ml noradrenaline	3.2	2.2	1.8
18		4.3	2.2	
		2.1	6.1	
18	10 μg/ml adrenaline	1.1	9.7	6.7
		3.9	4.3	
		0.7	11.7	
18	hypertonic NaCl[1]	1.1	8.4	9.9
		0.9	9.7	
		0.5	14.2	
18	10 μg/ml adrenaline and hypertonic NaCl[1]	0.4	11.8	13.0
		0.3	13.9	
		0.4	12.1	
18	10 μg/ml noradrenaline and hypertonic NaCl[1]	0.4	15.8	13.3
		0.4	10.7	

flasks at 43° it is seen that the potassium fall of the plasma after 1—1 $^1/_2$ hour has ceased, and an evident rise begins. If the plasma is made hypertonic the potassium absorption continues.

There may be many explanations of these results. One of them might be the removal of the adrenaline and noradrenaline. At any rate when the blood has been rocked for one and a half hour at 43° addition of very small amounts of the two substances will cause a diminished potassium rise or at higher concentrations a fall of the potassium concentrations of the plasma.

[1] added $^1/_{40}$ volume 1400 mM/l NaCl.

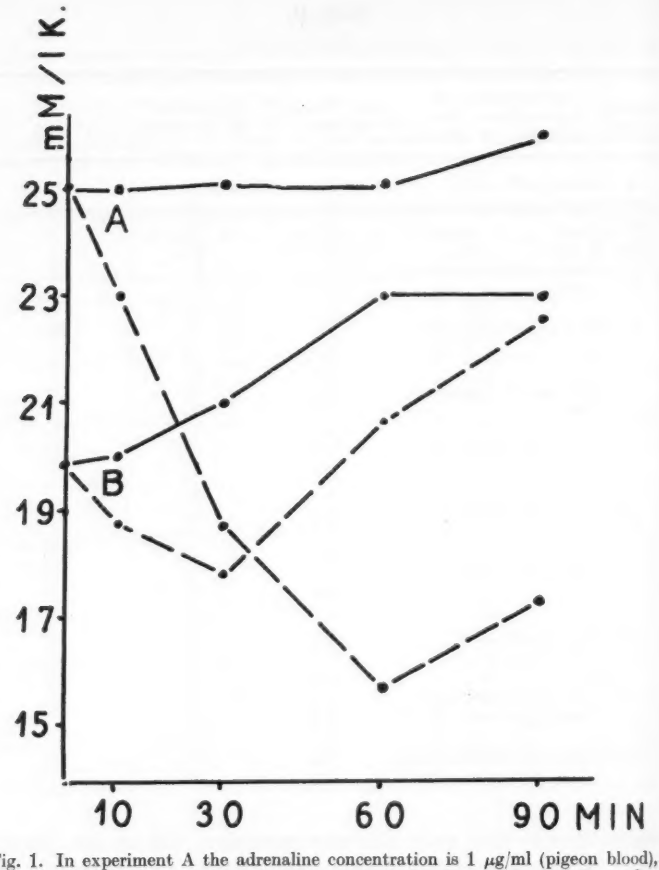

Fig. 1. In experiment A the adrenaline concentration is 1 μg/ml (pigeon blood), in B 0.01 μg/ml. ● —— ● —— ● —— = controls, ● - - - ● - - - ● = after adrenaline. Rocking at 43°, above-standing air 95 % O_2 and 5 % CO_2.

The difficulty in these experiments is that the control values of serum potassium are rising. In all experiments the blood has been rocking for one and a half hour at 43° (about 45 times a minute, excursions 25 degrees) in 50 ml Erlenmeier flasks closed with rubber stoppers. The air above the blood contained 95 % O_2 and 5 % CO_2, and every time a sample of blood has been taken, 100 ml of that air mixture is emptied into the flasks by means of a syringe.

Table III.

Pigeon.

Concentrations of Adrenaline and Noradrenaline in the Blood.

A = Adrenaline and the corresponding ciphers to the right are K-decrease in mM/l at the different concentrations of the plasma.
N = Noradrenaline and the corresponding ciphers to the right are K-decrease in mM/l at the different concentrations of the plasma.

Minutes at 43°		1 μg/ml	0.1 μg/ml	0.01 μg/ml	0.001 μg/ml	0.00033 μg/ml	0.00017 μg/ml	0.0001 μg/ml
30	A	3.2	3.0	2.1				
	N	3.4	3.4	2.3				
30	A		2.5	3.1	1.9			
	N		2.7	3.4	2.8			
30	A			3.0	0.5			0.1
	N			1.9	1.2			0.5
30	A			2.0	0.5			0.0
	N			1.7	1.1			0.3
30	A				0.7	0.2	0.0	
	N				1.6	0.5	0.4	
20	A				0.6	0.4	0.1	
	N				1.3	0.6	0.3	
10	A				0.5	0.3	0.2	
	N				1.1	0.7	0.5	
50	A				0.5	0.0	0.0	
	N				1.1	0.3	0.3	
30	A				0.1	0.0	—0.1	
	N				1.0	0.6	0.3	
Average	A	3.2	2.8	2.6	0.7	0.2	0.0	0.1
	N	3.4	3.1	2.4	1.4	0.5	0.4	0.4

Now equal parts of the blood are placed into two 50 ml Erlenmeier flasks, to the control is added $^1/_{50}$ volume 0.9 % NaCl, to the other $^1/_{50}$ volume 0.9 % NaCl + adrenaline. It is of great importance that the handling of the two flasks, and later the samples are exactly alike, as the tension of CO_2 and O_2 of the blood samples is of influence, and it seems that even shaking of the flask may be significant. In fig. 1 two experiments are shown, where the plasma potassium has been raised by addition of potassium chloride.

In experiment A the potassium concentration of the control keeps rather constant, in the flask with 1 μg/ml adrenaline the plasma potassium falls steeply during the first hour, and then rises. In experiment B the adrenaline concentration is 0.01 μg/ml. The fall of potassium has ceased after 30 minutes, the potassium then rises very steeply, so that the two curves nearly meet after 90 minutes.

The fall of plasma potassium in the experiments corresponds to a similar rise of cell potassium as the haematocrit-values of the pigeons lie about 40 to 50.

A special method has been used to measure the effect of adrenaline and noradrenaline at various concentrations. First the blood is rocked for one and a half hour at 43°. The above-standing air consists of 95 % O_2, 5 % CO_2. From the blood of each pigeon 9 samples (1 ml) are prepared 3 of which are controls. The controls are added $^1/_{50}$ volume 0.9 % NaCl, the interjacent samples the same + varying concentrations of adrenaline and noradrenaline. The controls are made before and after the other samples on account of the increasing plasma potassium. The whole procedure takes about 15 minutes. Twice during this time 100 ml of the air mixture are injected into the Erlenmeier flask, and when the centrifuge glasses are closed they are placed in water-bath at 43° for the time fixed without rocking, and then centrifuged. The method is gentle to the blood cells, and the oxygen content of the blood is adequate, but it must be admitted that the tension of oxygen of the blood is unknown.

The results are seen in table III. There is no doubt that noradrenaline has a stronger effect (two—three times) than adrenaline when weak solutions are used, but with strong solutions the effect is the same. Noradrenaline has a certain effect in concentrations of 0.001 μg/ml and probably down to 0.0001 μg/ml. As the substances used are synthetic[1], and contain d as well as l forms, and only the l form is active, the sensitivity of the cells is twice as high as the above-mentioned figures state.

As a method to determine the concentrations of the two substances it is as sensitive as any other biological method.

Discussion.

From the results of table III it appears that if the blood from a pigeon at the bleeding contains about 0.01 μg/ml of adrenaline or noradrenaline then a maximal decrease of the potassium concentration will be found, and if further quantities of these substances are added the only effect will be a prolonged potassium absorption of the blood cells.

How does adrenaline and noradrenaline act on the blood cells?

[1] Adrenaline from Usines Chimiques Des Laboratoires Français, Paris. Noradrenaline from Hoechst, Frankfurt.

It must at once be admitted that there are several possibilities. When adrenaline in the blood from the frog has comparatively much stronger effect than noradrenaline, and with pigeon cells the opposite is the case, though not as evident, it might indicate that there is more than one mode of action. Probably the frog cells have a low metabolism during the winter, and adrenaline has here the strongest effect, and corresponding to earlier observations with yeast cells potassium absorption was strongest when substances were added which increased the metabolism (ØRSKOV 1950). In some experiments the changes of the sodium concentration of the plasma after addition of adrenaline and noradrenaline have been determined. But nothing was found.

Two papers have been found which deal with the permeability of potassium and adrenaline.

GOFFART and PERRY (1951) have studied the effect of adrenaline, noradrenaline and isopropyl noradrenaline on the loss of ^{42}K from the isolated resting rat's diaphragma continuously irrigated with potassium free Tyrode's solution.

Rather concentrated solutions were used (10 μg/ml for nor- and adrenaline, and 50 μg/ml for isopropyl adrenaline). At first during the irrigation a diminished — later on an augmented loss of ^{42}K from the muscle was seen. Isopropyl noradrenaline was used because it has no vasoconstrictor action. Of course a diaphragma preparation is much more complicated than blood.

BORN and BÜLBRING (1955) using a preparation of taenia coli of the guinea pig find that adrenaline does not change the loss of ^{42}K from the muscles, but the uptake of ^{42}K is increased. Relaxation of the muscle during the experiment was necessary.

Also D'SILVA's observation (1936) that injection of 50—100 μg adrenaline i.v. in the cat produces a sudden rise of plasma potassium ought to be mentioned. The rise is derived from the liver, but the peculiar thing is that it lasts for a few minutes only.

Summary.

When the frogs are placed in ice-water for one hour and then bled, plasma potassium in most cases is increasing. Addition of adrenaline and noradrenaline make the blood cells absorb potassium.

Adrenaline has an effect down to 0.01 μg/ml, noradrenaline is from 10 to 100 times less efficient.

Hypertonic plasma plus adrenaline or noradrenaline produces extremely low potassium values (below 0.5 mM/l).

When pigeon blood is rocked at 43° for one and a half hour the plasma concentration is rising. The pigeon blood cells are now very sensitive to adrenaline and noradrenaline (noradrenaline 2—3 times more efficient).

Down to 0.001 μg/ml or perhaps even 0.0001 μg/ml of noradrenaline has an effect.

It is of importance that all the samples are dealt with in exactly the same way (dilution, shaking etc.)

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Potentiation by Histaminase Inhibitors of the Blood Pressure Responses to Histamine in Cats.

By

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The effects on the cat's blood pressure of histamine injected into the blood stream are usually transient. It is well known that the injected histamine rapidly disappears from the blood. EMMELIN (1951) working on cats found that the kidneys and the small intestine were especially potent in removing injected histamine from the circulating blood. These organs contain the greater part of the histaminase in the cat (HÆGER and KAHLSON 1952).

A number of substances are known to inhibit histaminase (diamine oxidase), (BLASCHKO, FASTIER and WAJDA 1950, ZELLER, BARSKY, FOUTS, KIRCHHEIMER and VAN ORDEN 1952, SCHULER 1952). SCHAYER, in studies on the metabolism of C^{14}-labelled histamine in intact animals, found that some of these inhibitors could prevent the breakdown of injected histamine (SCHAYER 1952 and 1953 a, SCHAYER, KENNEDY and SMILEY 1953 b).

MONGAR and SCHILD (1951) and ARUNLAKSHANA, MONGAR and SCHILD (1954) showed that histaminase inhibitors potentiate the effects of histamine on isolated organs from guinea pigs. They presented strong evidence that this potentiation was in fact due to histaminase inhibition by the drugs used. The same authors found that B_1-pyrimidine, a histaminase inhibitor, potentiated the effects of injected histamine on the blood pressure in four out of eleven eviscerated cats.

The purpose of the experiments reported here is to study by the use of histaminase inhibitors the rôle played by histaminase in the removal of injected histamine from the circulating blood.

The experimental technique is similar to that used by EMMELIN in 1951. It involves injections of histamine into the renal and femoral arteries and into the femoral vein. The histamine that escapes into the general circulation produces a fall in blood pressure, the magnitude of which is taken as a measure of the amount of escaped histamine. The effects of various histaminase inhibitors on the blood pressure responses to injected histamine have been studied.

Methods.

Sixty-four cats of both sexes, weighing 2—5 kg, were used. They were anaesthetized with ether followed by chloralose 70—80 mg per kg intravenously. The blood pressure in a carotid artery was measured with a mercury manometer. Injections were made through polythene tubes into the femoral vein and into branches of the femoral and renal arteries.

A branch of the femoral artery suitable for insertion of a cannula is usually found just below the inguinal ligament. The femoral vein was left intact on the side where injections into the femoral artery were made. The region of the left renal hilus was exposed through a midline abdominal incision. The renal artery usually divides into two branches just medially to the hilus. The ventral of these branches was cannulated. Most of the experiments were completed with an injection of indigo carmine dissolved in 0.9 per cent saline into the renal artery. The injected volume was equal to the volumes of the proceding injections of histamine. The kidney was removed immediately after the injection and the coloured part as well as the whole kidney were weighed. Sometimes we observed that the cortex had taken up less dye than usual even in that part of the kidney where we had expected an intact circulation. This might indicate a cortical ischaemia, induced by stimulation of the nerves to the kidney in the cannulation procedure. Since it would seem that a great proportion of the histaminase in the kidney is present in the cortex (ZELLER 1942) we thought it desirable to try to prevent the development of such vascular changes by blocking the nerves to the kidney by infiltrating the perihilar tissues with 1 ml 2 % lidocaine before the cannulation of the renal artery. After adopting this procedure we have not observed any cortical ischaemia as judged by the results of injection of indigo carmine.

It is known that histamine liberates pressor amines from the adrenal medulla. Histaminase inhibitors injected in the renal artery might reach the adrenal gland on the same side and influence the release of pressor amines in response to histamine injected by the same route. In four cats, one experiment with each inhibitor, the left adrenal gland was removed before the renal artery on the same side was cannulated.

The histamine inactivating capacity in extracts from organs removed during or at the end of the experiments was determined by the method used by HÆGER and KAHLSON 1952 a.

Histamine acid phosphate, β-(2-pyridyl)-ethylamine dihydrochloride and acetylcholine chloride were dissolved in 0.9 per cent saline immediately before the experiment and injected by hand with a 0.5 ml tuberculin syringe connected to the polythene tube by a stainless steel cannula of suitable size. The volume injected was 0.1—0.2 ml followed by 0.2 ml to rinse the tube. The concentrations were so chosen that approximately equal volumes of the solutions of histamine, β-(2-pyridyl)-ethylamine and acetylcholine were needed for equal responses of the blood pressure. The injection rates were standardized with a stop watch. The histaminase inhibitors were administered either intravenously or intraarterially, single doses or long time infusions being used.

The following substances have been used: Histamine acid phosphate (The British Drug Houses Ltd.) β-(2-pyridyl)-ethylamine dihydrochloride (Maltbie Chemical Laboratories, Newark, New Jersey), acetylcholine chloride (Hoffman-La Roche & Co AG).

The following inhibitors have been used: 2-methyl-4-amino-5-aminomethylpyrimidine (B_1-pyrimidine) and isonicotinylhydrazine (INH) (Hoffman-La Roche & Co AG), aminoguanidine hydrocarbonat (Fluka AG), stilbamidine isethionate (May & Baker Ltd).

The concentrations of all substances refer to the salts, except for histamine and isonicotinylhydrazine where they refer to the base. All concentrations are expressed in weight per volume unless otherwise stated.

Results.

Blood Pressure Responses to Histamine Injected by Various Routes.

When given by vein 0.05—0.25 μg per cat were required to produce a distinct fall in arterial blood pressure. The fall occurred 12—18 seconds after the beginning of the injection. When injected into the femoral artery 0.1—0.4 μg were needed to produce a depressor response. With 0.2—0.4 μg this depressor response was usually diphasic consisting of an initial depression which occurred 5—6 seconds after the beginning of the injection followed by a delayed response which occurred after 14—20 seconds. The delayed response disappeared if the blood from the femoral vein was withdrawn during the injection of histamine and replaced by infusion of histamine-free blood. When injected into the renal artery the dose of histamine required to produce a distinct fall in blood pressure was 0.8—6.0 μg. The latency of the response was about the same as with injections into the femoral vein.

The depressor response disappeared when the blood from the renal vein was withdrawn during the injection of histamine and replaced with infusion of histamine free blood.

The relations between the amounts of histamine producing equal falls in arterial blood pressure after injections in the renal artery, femoral artery and femoral vein were on the average 25 : 2 : 1 respectively.

Effects of Histaminase Inhibitors.

All four inhibitors can by themselves produce a fall in blood pressure. This is especially noticeable with stilbamidine. According to MacIntosh and Paton (1949) the depressor effects of stilbamidine are due to the liberation of histamine. In some of the experiments by Arunlakshana, Mongar and Schild B_1-pyrimidine caused a fall in blood pressure in eviscerated cats. These authors found that depression of blood pressure by the inhibitor and potentiation generally were associated. "In several instances B_1-pyrimidine caused neither a depression of blood pressure nor potentiation."

In the present experiments the rates of injection and the amounts injected were such that the inhibitors had no observable effects of their own on the blood pressure.

A. Effects of inhibitors on the blood pressure responses to histamine injected into the femoral vein.

No potentiation of the depressor responses to histamine injected in the femoral vein was observed. This has been studied in nine cats after administration of stilbamidine, in five after B_1-pyrimidine, in two after isonicotinylhydrazine and in six after aminoguanidine. One typical experiment is shown in fig. 1. Aminoguanidine 0.2 mg per minute per kg body weight was given by vein during 50 minutes, totalling 50 mg or 10 mg per kg b. w. The doses were large enough to produce a potentiation of the effects of histamine given in the renal artery. In some experiments of this type the kidneys were removed and examined for histamine inactivating capacity. Under the influence of the injected inhibitor the histamine inactivating capacity in crude extracts from these organs was less than 10 per cent of the normal. The kidneys had been removed within twenty minutes after the administration of the inhibitors.

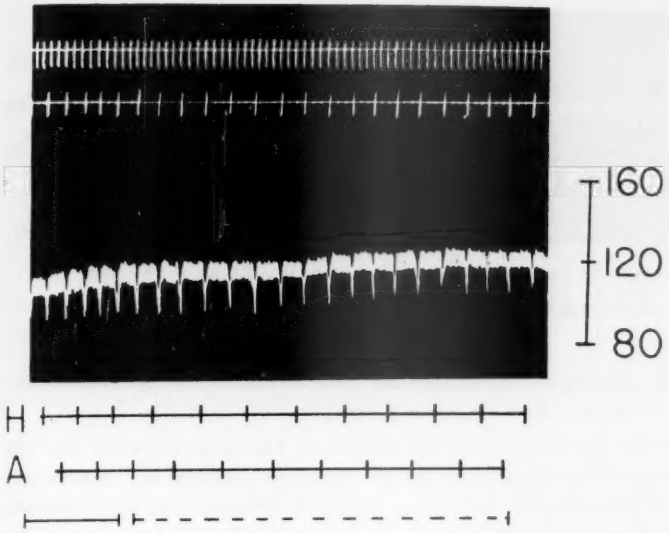

Fig. 1. ♂ 5.1 kg. From above: time in minutes, injections in the right femoral vein, blood pressure. H = 0.1 μg of histamine, A = 0.03 μg of acetylcholine. Continuous infusion in the left femoral vein of saline |——| and aminoguanidine 1 mg per minute. |- - - -|

Table 1.

Cat No.	Weight in kg	Inhibitor	Total dose of inhibitor in mg	μg inactivated histamine per g tissue per hour
P 1	3.6	aminoguanidine	18	0
P 7	4.4	"	50	0
P 14	5.1	"	50	0
P 4	4.4	B_1-pyrimidine	26	2
P 15	3.0	"	30	5
P 2	4.2	isonicotinyl-hydrazine	24	2
P 16	3.2	—	—	80
P 58	4.0	—	—	56

P 16 and P 58 were submitted to the same experimental procedures as the other cats but received no inhibitor. The figure for histaminolytic activity in extracts from normal kidneys is 97.9 ± 5.3 μg per gram tissue per hour (Hæger, Jacobsohn and Kahlson 1952).

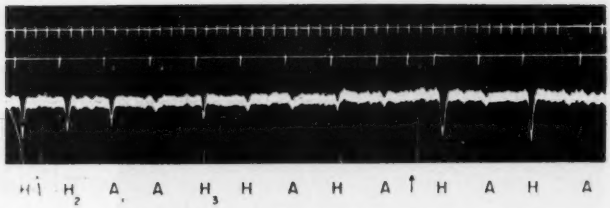

Fig. 2. ♂ 3.6 kg. From above: time in 30 sec., injections in the renal artery, blood pressure.
$H_1 = 5.0$ μg, $H_2 = 4.0$ μg, $H_3 = 3.2$ μg, $H = 2{,}8$ μg of histamine.
$A_1 = 1.0$ μg and $A = 0.8$ μg of acetylcholine.
Arrow indicates injection in the renal artery of 500 μg aminoguanidine.

B. Effects of inhibitors on the blood pressure responses to histamine injected into the femoral artery.

No potentiation of the effects of histamine injected into the femoral artery was observed. This series comprises 1 experiment with each of the following inhibitors: Stilbamidine, B_1-pyrimidine and isonicotinylhydrazine, and 3 experiments with aminoguanidine. The doses were the same as those used in the experiments with injections into the femoral vein.

C. Effects of inhibitors, on the blood pressure responses to histamine injected into the renal artery.

The depressor responses to histamine injected into the renal artery were regularly potentiated after the administration of inhibitors in suitable doses.

The doses of inhibitors required for potentiation were as follows:

1. *Aminoguanidine:* Fig. 2. In our experiments the smallest potentiating dose of aminoguanidine given in single injection into the renal artery was 0.01 mg. The potentiation after 0.1 mg aminoguanidine was usually maximal since no further increase in degree of potentiation could be produced by tenfold increase in the dose of inhibitor. The potentiating effect of 0.3 mg aminoguanidine lasted at least half an hour which was the longest observation period in these experiments.

Intravenous infusion of 0.2 mg aminoguanidine per minute per kg b. w. potentiated the effects of histamine given in the renal artery but not those of histamine given in the femoral artery.

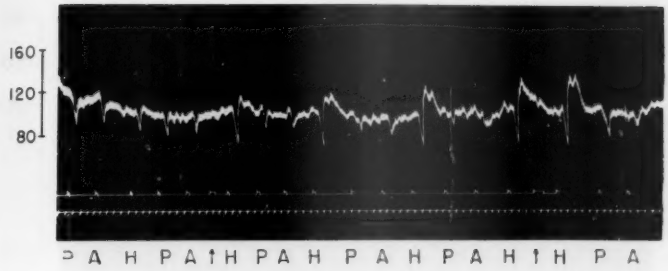

Fig. 3. ♀ 4.1 kg. left adrenal gland removed. From above: Blood pressure, injections in the renal artery, time in 30 sec.
P = 130 μg β-2-pyridylethylamine, A = 1.0 μg acetylcholine,
H = 4.0 μg histamine.
First arrow indicates injection in the renal artery of 2 mg isonicotinylhydrazine and second arrow 10 mg isonicotinylhydrazine.

2. *B_1-pyrimidine:* The smallest dose of B_1-pyrimidine which produced a potentiation when given in a single injection into the renal artery was 0.1 mg. Doses of 1 mg regularly potentiated the effects of histamine for at least 10 minutes.

With prolonged infusion into the femoral vein of 0.1 mg per minute per kg b. w. B_1-pyrimidine potentiated the effects of histamine given in the renal artery but not those of histamine given in the femoral artery.

3. *Isonicotinylhydrazine:* Fig. 3. The smallest potentiating dose of INH given in a single injection into the renal artery was 1 mg. The effect lasted more than 10 minutes. After 3 mg of INH the potentiation lasted at least 30 minutes.

In continuous intravenous infusion of 0.6 mg per minute per kg b. w. INH potentiated the effects of histamine injected into the renal artery but not those of histamine injected into the femoral artery.

4. *Stilbamidine:* Fig. 4. When given in a single injection into the renal artery 0.25—0.5 mg per cat were required to produce a potentiation, which would last 2—5 minutes. The effect of 2 mg lasted about 10 minutes.

In prolonged infusion into the femoral vein (0.1 mg per minute per kg b. w.) stilbamidine potentiated the effects of histamine in the renal artery but not those of histamine injected into the femoral artery.

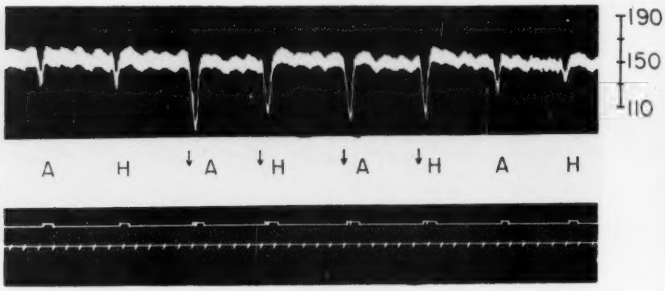

Fig. 4. ♂ 3.2 kg. From above: Blood pressure, injections in the renal artery, time in 30 sec. H = histamine 1.0 μg and A = acetylcholine 2.0 μg. Arrows indicate injections of stilbamidine 0.25 mg.

After aminoguanidine, isonicotinylhydrazine or B_1-pyrimidine the doses of histamine which produced equal falls in blood pressure when given in the renal artery and in the femoral vein were approximately 10 : 1 against 25 : 1 before the administration of the inhibitor.

D. The influence of histaminase inhibitors on renal blood flow.

It seemed possible that an increase in the rate of blood flow through the kidney might reduce the time available for the removal of histamine from the blood by the kidney and thus produce a potentiation of the effects of histamine injected into the renal artery. Accordingly the effects of histaminase inhibitors on the rate of blood flow through the kidney was studied. The technique was the same as in the experiments on potentiation except that the blood from the renal vein was drained through a drop recorder, which operated an ordinate writer (Clementz and Ryberg 1949). The results are shown in fig. 5. All the inhibitors increased renal blood flow. With the doses used the longest duration of the increase occurred after stilbamidine. After 2 mg of stilbamidine the increase lasted less than 3 minutes. It thus seems unlikely that the histamine potentiations observed in our experiments are due to increased renal blood flow. This assumption is further strengthened by the absence of potentiation of the effects of β-(2-pyridyl)-ethylamine and acetylcholine after administration of B_1-pyrimidine, isonicotinylhydrazine or aminoguanidine.

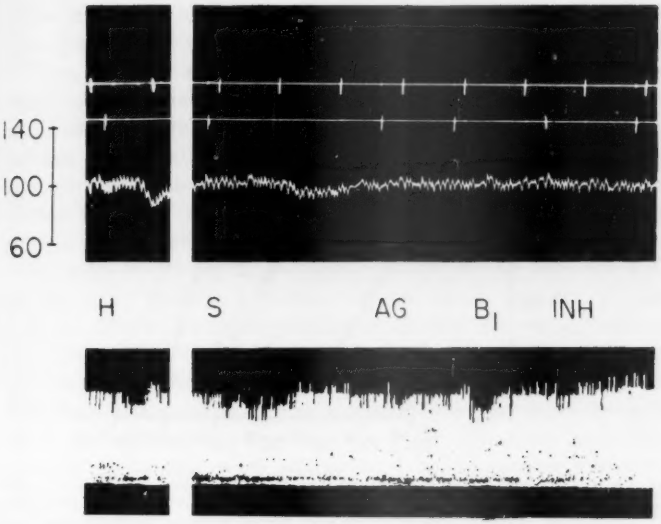

Fig. 5. ♂ 1.8 kg. From above: time in minutes, injections in the renal artery, blood flow (height of ordinates directly proportional to the time intervals between the drops with a maximal error of 15 per cent.)
H = 2.0 μg histamine, S = 2.0 mg stilbamadine, AG = 200 μg aminoguanidine, B^1 = 2.0 mg B_1-pyrimidine, INH = 2.0 mg isonicotinylhydrazine.

E. In vitro inhibition of histaminase.

In order to get some information on the relative inhibiting potencies in vitro of our inhibitors we have incubated a crude extract of kidneys from normal cats with histamine and studied the influence of various concentrations of inhibitors on the inactivation of histamine. Histamine and inhibitor were added simultaneously to the enzyme extract. The initial concentration of histamine in all incubations was $10^{-5.7}$ M. The inactivation of histamine was assessed by assay on the guinea pig's ileum. The results are shown in fig. 6.

The Specificity of the Potentiation of Histamine Effects by Histaminase Inhibitors.

A. β-(2-pyridyl)-ethylamine.

β-(2-pyridyl)-ethylamine was shown by WALTER, HUNT and FOSBINDER in 1941 to have an action similar to histamine on

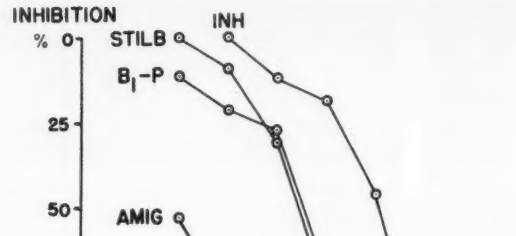

Fig. 6. Relations between the inhibiting capacities in vitro of AMIG = aminoguanidine, B_1-P = B_1-pyrimidine, STILB = Stilbamidine and INH = Isonicotinylhydrazine. Crude extract of pooled kidneys from normal adult cats incubated with histamine in a concentration of 0.2 μg/ml $10^{-5.7}$ M.

isolated strips of guinea pig's intestine. LEE and JONES (1949) studied the activity of some β-aminoethyl heterocyclic nitrogen compounds on the guinea pig's ileum and cat's blood pressure. Among the compounds which had a histamine-like activity was β-(2-pyridyl)-ethylamine. The specificity of the activity was tested by a histamine antagonist. ARUNLAKSHANA (1953) found that the histamine-like actions of β-(2-pyridyl)-ethylamine were antagonized by antihistamines to the same extent as the actions of histamine itself. "The results of ARUNLAKSHANA suggest that β-(2-pyridyl)-ethylamine acts on the same receptors as histamine itself" (ARUNLAKSHANA, MONGAR and SCHILD 1954). According to WERLE and PALM (1953) β-(2-pyridyl)-ethylamine is attacked by diamine oxidase from pea seedlings. The oxygen consumption was rather small compared to that of histamine under the same experimental conditions. ARUNLAKSHANA, MONGAR and SCHILD 1954 worked with histaminase in the form of a crude acetone dried powder prepared from pig kidney cortex. As judged by measurement of biological activity and ammonia production β-(2-pyridyl)-ethylamine was not attacked by histaminase from this source. The same authors found that the effect of β-(2-pyri-

dyl)-ethylamine on the isolated ileum and the tracheal chain from guinea pigs were not potentiated by histaminase inhibitors.

Thus it would seem that β-(2-pyridyl)-ethylamine acts on the same receptors as histamine but is much more slowly or not at all inactivated by histaminase. This compound was used in the present investigation as a tool to differentiate between a potentiation due to inhibition of histaminase and a potentiation caused by other mechanisms, in the former case the responses to β-(2-pyridyl)-ethylamine should not be potentiated.

By vein 3—20 μg of β-(2-pyridyl)-ethylamine per cat were necessary to produce a distinct fall in blood pressure. In the femoral artery the corresponding dose was 20—80 μg and in the renal artery 40—200 μg. Diphasic depressor responses were observed where the depressor responses to histamine were diphasic. The time relationship of the response was the same as with histamine. The dose response curves for histamine and β-(2-pyridyl)-ethylamine were similar and the depressor responses were equally diminished after mepyramine.

The relative doses of β-(2-pyridyl)-ethylamine required to produce equal falls of the blood pressure when injected in the renal artery, the femoral artery and the femoral vein were approximately 9 : 3 : 1. None of the inhibitors potentiated the depressor responses to β-(2-pyridyl)-ethylamine given in the renal artery (fig. 3).

B. Acetylcholine.

The effects of acetylcholine were not potentiated by histaminase inhibitors in the experiments by ARUNLAKSHANA, MONGAR and SCHILD (1954). This substance was used in some of the experiments presented here as a further guide as to the specificity of the potentiations studied.

By vein 0.02—0.1 μg per cat were needed to produce a distinct fall in blood pressure. In the femoral artery the corresponding dose was 0.15—0.80 μg and in the renal artery 0.2—2.0 μg. The relations between equiactive doses of acetylcholine injected in the renal artery, the femoral artery and the femoral vein were approximately 20 : 6 : 1 respectively.

B_1-pyrimidine, isonicotinylhydrazine and aminoguanidine did not potentiate the depressor effects of acetylcholine given in the renal artery (fig. 2). Stilbamidine, however, potentiated the effects of acetylcholine given in the renal artery (fig. 4).

Discussion.

It seems a priori likely that the mechanism responsible for the fall in blood pressure after injection of histamine in the renal artery and in the femoral vein is the same. This assumption is supported by the following observations. In experiments where the blood from the renal vein was withdrawn and substituted for there was no fall in blood pressure on injection of histamine in the renal artery. In the experiments where renal blood flow was measured there was no fall in arterial blood pressure concomitant with the vasodilatation in the kidney. The fall in blood pressure was delayed due to the passage of the blood through the flow recorder. At the time of the fall in arterial blood pressure there was a decrease in renal blood flow (fig. 5).

The depressor response to histamine injected into the femoral artery was usually diphasic, the initial fall ensuing 5—6 seconds after the injection is probably due to vasodilatation in the leg. The consecutive fall disappeared if the blood from the femoral vein was withdrawn and substituted by histamine free blood.

It thus seems likely that the depressor responses to histamine injected in the femoral vein and in the renal artery as well as the second phase of the depressor response to histamine injected in the femoral artery are produced in the same way, that is by histamine reaching the caval vein and carried out in the general circulation. The blood pressure responses to β-(2-pyridyl)-ethylamine were of the same type and time course as those to histamine.

The administration of histaminase inhibitors either by vein or in the femoral artery did not potentiate the depressor responses to histamine given by the same routes.

Each of the four inhibitors used in this investigation potentiated the depressor response to histamine injected in the renal artery. This potentiation might be due to an action of the drug on the receptors concerned in eliciting the fall in blood pressure. This is unlikely since the depressor responses to histamine injected in the femoral artery or vein were not potentiated. Moreover the effect of β-(2-pyridyl)-ethylamine, which is supposed to act on the same receptors as histamine, were not potentiated. Further the possibility was considered that the inhibitor injected in the renal artery reached the adrenal gland on this side and might reduce the release of pressor amines caused by the histamine

injected by the same route. In four experiments the left adrenal gland was removed. This did not change the potentiations of histamine effects after administration of inhibitors.

It seems very likely that the potentiation is due to an effect of the inhibitors on the kidney. An increase in the rate of blood flow through the kidney might reduce the time available for removal of histamine from the blood during its passage through the kidney and thus increase the amount of histamine which escapes into the general circulation. All four inhibitors in the doses used increased renal blood flow, but the increase was slight and transient. Thus with 0.2 mg aminoguanidine the increase lasted less than 1 minute, whereas the potentiation after this dose would last at least 20 minutes. Moreover, a potentiation produced in this way should also affect β-(2-pyridyl)-ethylamine and acetylcholine.

Next to consider is the capacity of extracts from feline renal tissue to inactivate added histamine, demonstrated by HOLTZ, HEISE and SPREYER (1938) and HÆGER, JACOBSOHN and KAHLSON (1952). This capacity is probably due to the enzyme histaminase since it can be destroyed by boiling, inhibited by histaminase inhibitors and since it is associated with an oxygen consumption, that runs parallel with the biological inactivation (LINDAHL, LINDELL, WESTLING and WHITE to be published). The presence in the kidney of histaminase suggests that the potentiation of the depressor response to histamine injected in the renal artery after administration of histaminase inhibitors is due to the inhibition of this enzyme. This assumption is supported by the following observations:

There was no potentiation of the effects of a histamine analogue which according to ARUNLAKSHANA, MONGAR and SCHILD acts on the same receptors as histamine but is not inactivated by histaminase. The effects of acetylcholine were not potentiated by B_1-pyrimidine, isonicotinylhydrazine or aminoguanidine. Stilbamidine, however, potentiated the depressor response to acetylcholine injected in the renal artery. The mechanism of this potentiation is not known. However, it may be mentioned that stilbamidine inhibits cholinesterase (BERGMANN, WILSON and NACHMANSOHN 1950).

The depressor effect of histamine given in the renal artery was potentiated but the effect of histamine injected in the femoral artery was not. The renal tissue is rich in histaminase, whereas

the histamine inactivating capacity of crude extracts from the tissues in the hindleg is small. (HÆGER and KAHLSON 1952 b, and unpublished results from this laboratory.)

The technique used in this investigation does not allow a detailed quantitative analysis of the results. However, it was usually possible to estimate the degree of potentiation by comparing the relations between equiactive doses of histamine given in the renal artery and in the femoral vein before and after the administration of inhibitors. These relations averaged 25 : 1 before the injection of the inhibitor. The greatest potentiations observed after administration of aminoguanidine, B_1-pyrimidine and isonicotinylhydrazine were such that the same relations were approximately 10 : 1, indicating a reduction by around 60 per cent in the kidney's capacity to remove histamine from the blood. No further reduction in this capacity was seen even after a tenfold increase in the dose of inhibitor. This seems to indicate that the inhibitors act through a common mechanism and that the greatest potentiation which is obtainable is determined by this mechanism and not by the dose of inhibitor.

Aminoguanidine was the most effective and isonicotinylhydrazine the least effective potentiator. According to SCHULER (1952) aminoguanidine is the most potent inhibitor of diamine oxydase (histaminase). SCHAYER (1953 b) found aminoguanidine several times as active as isonicotinylhydrazine in vivo. In this laboratory we have incubated crude extracts from cat's kidneys with histamine and studied the influence of some inhibitors on the inactivation of histamine. As can be seen in fig. 6 aminoguanidine was the most potent inhibitor and isonicotinylhydrazine the least potent, while the inhibiting capacities of B_1-pyrimidine and stilbamidine were intermediate. As judged by the smallest doses of inhibitors that potentiated the histamine effects in our experiments there is a parallelism between the potentiating and the inhibiting capacities of these inhibitors.

Finally, in cats which had been given inhibitors in doses sufficient to produce maximum potentiation there was no appreciable histamine inactivating capacity in extracts from the kidneys removed within 20 minutes after the injection of the inhibitor.

The capacity of the feline kidney to remove histamine from the blood was first demonstrated by EMMELIN. This capacity which we have confirmed, can be reduced by about 60 per cent by histaminase inhibitors. SCHAYER, KENNEDY and SMILEY (1953 b)

studied the pattern of C^{14}-histamine metabolites in paper chromatograms of urine from kittens. From the changes in this pattern produced by administration of aminoguanidine or Marsilid to the animals they conclude that an enzyme (or enzymes) different from diamine oxidase plays the major rôle in the metabolism of histamine in cats. This enzyme (enzymes) was given the name Histamine metabolizing enzyme II. It is possible that this enzyme is connected with that capacity of the kidney to remove histamine from the blood, which remains after the administration of histaminase inhibitors.

EMMELIN found that some of the histamine which had been taken up by the kidney remained there for some time and that some was excreted in the urine. The extent to which these events are modified by histaminase inhibitors is not known.

Summary.

1. The effects on the cat's arterial blood pressure of histamine, β-(2-pyridyl)-ethylamine and acetylcholine injected into the femoral vein, femoral artery and renal artery have been studied.

2. As judged by the relative effectiveness of these injections the cat's kidney has a considerable capacity to remove injected histamine from the circulating blood.

3. After the administration of histaminase inhibitors the depressor response to histamine injected into the renal artery was potentiated.

4. Evidence is presented that this potentiation is due to the inhibition of the histaminase in the kidney.

5. The maximum potentiation obtained in these experiments would correspond to an approximate reduction by 60 per cent of the kidney's capacity to remove injected histamine from the blood.

6. No potentiation of the effect of histamine injected into the femoral artery or vein was observed.

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The Content of Carbohydrates in Caseins from Different Species.

By

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Comparative investigations have revealed striking differences between human and cow's casein concerning physico-chemical and biological properties (MELLANDER 1947). However, the differences in chemical composition so far reported have been limited to a significantly higher phosphorus content in cow's casein (see MELLANDER 1947). WILLIAMSON (1944) has also found a slight dissimilarity in amino acid composition, using microbiological techniques. Reinvestigations of the amino acid content by means of ion-exchange chromatography by the method of MOORE and STEIN (1951) have also demonstrated minor differences (JOHANSSON, STRID and MELLANDER, unpublished experiments).

In a preliminary communication we have drawn attention to the appreciably higher carbohydrate content of human casein (CRAVIOTO, JOHANSSON and SVENNERHOLM 1955).

No detailed investigation of the carbohydrate content of caseins has been performed earlier. Some researchers have used cow's casein when applying ELSON and MORGAN's method (1933) for hexosamine determinations on protein hydrolysates (NILSSON 1936, MASAMUNE and NAGAZUMI 1937, ANASTASSIADIS and COMMON 1953). Hexose was analysed in some casein preparations by SÖRENSEN and HAUGAARD in 1933. MASAMUNE and MAKI (1952) prepared a mucopolysaccharide from a tryptic digest of cow casein. It contained glucosamine, galactose and mannose. Despite

thorough perusal of the literature we have found no analysis of the carbohydrates in human casein.

In the present study we have estimated the amount of hexose, hexosamine and sialic acid in caseins from bovine and human milk. On account of the decidedly larger amount of carbohydrates found in human casein, we were interested to see if human casein was unique in this respect. Therefore we have also included analyses of caseins from sheep, goat, horse, whale and reindeer.

Experimental.

Materials. In all cases the milks used for the preparation of the caseins were as fresh as possible. The milk from whale and reindeer was frozen and toluol added for preservation. Mature milk was used with the exception of horse where the casein was prepared from colostrum. No data were available for the whale milk.

Preparation of caseins. Caseins from all species except homo could be precipitated in the same way by merely acidifying the milk to pH 4.6 with 0.1 N hydrochloric acid with vigorous stirring. For the sake of convenience, however, the milk from whale and reindeer was diluted twice with distilled water owing to their large amount of solids. In the case of human milk the standard method for the preparation of casein could not be used. It was found necessary either to dilute the milk 5—10 times or to remove calcium ions by potassium oxalate before adding hydrochloric acid. The precipitates of human casein had a slight but obvious mucinous character.

The precipitated caseins were washed first with physiological saline and afterwards repeatedly with distilled water. The wash water was tested for reducing sugar. At least two washings were given after the wash water was found to be negative. The caseins were dried with ethanol and the ethanol removed by ether. They were dried to constant weight above phosphorus pentoxide in a vacuum desiccator. Weighed amounts of caseins were dissolved in 0.1 N sodium hydroxide and aliquots taken for the carbohydrate analysis.

Methods.

Hexose was determined by an orcinol-sulphuric acid method as described by VASSEUR (1948). As standard was used an equimolar solution of galactose-mannose. By means of a sample blank the influence of sialic acid on the read absorbance could be eliminated (SVENNERHOLM 1956).

Hexosamine was determined by a modification of ELSON and MORGAN's method (SVENNERHOLM in manuscript), in which the influence on colour formation of interaction between amino acids and hexoses is negligible.

Sialic acid was determined with Bial's reagent (orcinol-hydrochloric acid + Fe^{3+}) by the method of SVENNERHOLM (in manuscript). As standard substance was used sialic acid from bovine submaxillary mucin, which was a gift from Professor GUNNAR BLIX, Uppsala, to one of us (L. S.).

Results and Discussion.

A comparison between the carbohydrate content of human and cow casein is given in Table I. In human casein there is a markedly greater amount of every component analysed — the difference being most pronounced for hexose and hexosamine.

Table I.

Carbohydrate Content of Human and Cow Caseins. (Values given in per cent of dry weight.)

Casein	Hexosamine	Hexose	Sialic Acid ($C_{13}H_{21}NO_{10}$'H_2O)	Total Carbohydrates
Human	[1] 1.32	1.98	0.76	4.03
[2](5)	[3] (1.15—1.61)	(1.76—2.40)	(0.74—0.79)	(3.63—4.79)
Cow	0.18	0.24	0.39	0.80
(5)	(0.15—0.20)	(0.22—0.24)	(0.31—0.51)	(0.70—0.93)

The total carbohydrate content is no less than about five times greater in human than in bovine casein. The small deviations inside the two groups are apparent. The bovine milk was from the dairy, so that no conclusions can be drawn about individual variations, but three of the five human caseins were prepared from the milk of different individual women. Here too the different preparations contained about the same amounts. These results demonstrate that it is possible to prepare caseins with a fairly constant carbohydrate content and that the individual variations also seem to be small.

The agreement with earlier results for cow casein is also good. SÖRENSEN and HAUGAARD (1933) found 0.31 % hexose, but they used a casein which was reprecipitated twice. We have also reprecipitated caseins several times as described for the preparation of α-casein by WARNER (1944), which led to a casein with twice the amount of hexose (0.42 %). TILLMAN and PHILIPPI

[1] Mean value.
[2] Number of samples.
[3] Range.

Table II.

Carbohydrate Content of Caseins from Different Species. (Values given in per cent of dry weight.)

Casein	Hexosamine	Hexose	Sialic Acid ($C_{13}H_{21}NO_{10}\cdot H_2O$)	Total Carbohydrates
Human	1.32	1.98	0.76	4.03
Cow	0.18	0.24	0.39	0.80
Sheep	0.15	0.23	0.11	0.55
Goat	0.16	0.22	0.30	0.68
Whale	0.42	0.59	0.37	1.38
Horse	0.44	0.55	0.56	1.55
Reindeer	0.23	0.44	0.46	1.13

(1929) also found a higher hexose value in caseins which had been reprecipitated several times (0.36 % and 0.49 %). We think however that our method of preparation will give a truer picture of the carbohydrate content of caseins in the natural state.

The hexosamine content reported by MASAMUNE and NAGAZUMI (1937) was 0.20 %—0.26 %, although NILSSON's (1936) figures were somewhat higher, 0.32 % and 0.40 %. ANASTASSIADIS and COMMON (1953), on the other hand, could not demonstrate hexosamine in bovine casein. We have not tried to isolate hexosamine from casein but its presence is indubitable. Chromatography on Dowex 50 of an acid hydrolysate of human casein by the method of GARDELL (1953) demonstrated two fractions, which gave a positive ELSON and MORGAN reaction for hexosamine. The faster-running fraction was eluted in the same position as crystalline glucosamine-HCl, and the slower-running in that of galactosamine-HCl. The ratio of glucosamine to galactosamine was found to be 2 to 1.

The chromogen reacting with Bial's reagent to give a red violet colour was isolated from bovine colostrum by KUHN and BROSSMER (1954), who named it lactaminic acid. It has also been isolated by ZILLIKEN, BRAUN and GYÖRGY (1955) from the non protein part of human milk under the name of gynaminic acid. We have preferred to use the name sialic acid proposed by BLIX, SVENNERHOLM and WERNER (1952), because BLIX (1936) was the first to isolate a pure substance in this group of chromogens. The most thoroughly investigated sialic acids have been

isolated from submaxillary mucin of different species (BLIX, LINDBERG, ODIN and WERNER 1955), where they have indicated different acyl-groups in the acid: in cow sialic acid an O-acetyl group and an N-acetyl group, in ovine sialic acid an N-acetyl group, and in porcine sialic acid an N-glycolyl group. Sialic acids isolated from other sources contain only an N-acetyl group as ovine sialic acid (ODIN 1955). Gynaminic acid isolated as described by ZILLIKEN, BRAUN and GYÖRGY (1955) gave an X-ray powder diagram identical with ovine sialic acid (SVENNERHOLM, unpublished results), which further supports the use of the name sialic acid for the chromogen of caseins.

All values given for sialic acid refer to a standard of bovine sialic acid isolated from cow submaxillary mucin and containing two acetyl groups. KUHN and BROSSMER (1956) have also isolated from milk of homo, cow, goat, sheep and pig, a carbohydrate moiety composed of sialic acid and lactose, in which the sialic acid contained an N-acetyl group and an O-acetyl group.

The physiological importance of the carbohydrate components of milk is still unknown. Sialic acid containing mucoproteins and mucopolysaccharides from other sources, such as *e. g.* urine (TAMM and HORSFALL 1950, 1952) and submaxillary mucin (BURNET 1954, KLENK and FAILLARD 1954) has been demonstrated to be the receptor substance of influenza virus. ZILLIKEN, BRAUN and GYÖRGY (1955) have also demonstrated that saccharides containing sialic acid (gynaminic acid) are a growth factor for Lactobacillus bifidus var. Penn.

Summary.

Caseins from milks of homo, cow, goat, sheep, horse, reindeer and whale have been investigated for their content of hexose, hexosamine and sialic acid.

The total carbohydrate content does not exceed 2 % except in the case of human casein, which contains about 4 %. The amount of all components investigated is greater in human casein, the difference in comparison with other species investigated being most pronounced for hexose and hexosamine.

We are indebted to the Head of the Department, Professor OLOF MELLANDER, for the gift of samples of bovine and human caseins and valuable discussions and criticism during the per-

formance of the work. We also owe our thanks to Professor JOHAN T. RUUD, Biologisk Laboratorium, University of Oslo, for a sample of whale milk.

We are also very grateful to Mrs. BIRGIT JOHANSSON for valuable technical assistance.

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A Further Analysis of the Neuromuscular Block Caused by Acetylcholine.

By

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It has been shown that persisting high concentrations of acetylcholine in the isolated frog skeletal muscle cause a neuromuscular block that is *not* associated with membrane depolarization, but is characterized by unreactivity of the end-plate region to the depolarizing effect of the transmitter substance (FATT 1954 and THESLEFF 1955). This was shown by the observation that the depolarization of the end-plate regions caused by acetylcholine was short-lasting, and that the neuromuscular block persisted when the resting membrane potential of these regions was completely repolarized.

As shown by FATT and KATZ (1951), depolarization of the end-plate region by the neuromuscular transmitter is due to a general increase in the membrane permeability of this region to *all* ions. According to this hypothesis, there are two alternative mechanisms to account for the observed repolarization of the end-plate region in the presence of persisting high concentrations of acetylcholine.

(1) Tubocurarine and certain quaternary ammonium compounds block neuromuscular transmission by decreasing the sensitivity of the end-plate "receptors" to the depolarizing effect of acetylcholine without affecting the ionic permeability of the membrane (FATT and KATZ). It is conceivable that persisting high concentrations of acetylcholine have a similar effect.

(2) Hypothetically, the general increase in ionic permeability of the membrane at the end-plate region could change to a state

in which only a high permeability to the smaller potassium and chloride ions remained. The possibility that the membrane was left highly permeable to potassium and chloride but not to sodium ions would explain the neuromuscular block and the repolarization of the membrane to a potential about equal to the normal resting potential (HODGKIN 1951).

Even if there is no reason to believe that the depolarization caused by acetylcholine at the end-plate region is due to an activation of carrying mechanisms for specific ions, as in the nerve membrane (FATT and KATZ), the experiments of HODGKIN and HUXLEY (1952) on the squid giant axon provide an interesting analogy to the aforementioned hypothesis. They showed that when the membrane potential of the axon was "clamped" at a low voltage, the membrane permeability to sodium and potassium ions increased. The sodium conductance was, however, brief, probably due to the inactivation of its carrying mechanism, whereas the high conductance to potassium ions was maintained.

With an action of acetylcholine similar to that of tubocurarine (1), the transverse electric resistance of the muscle membrane at the end-plate region would be normal, whereas if the block were due to a permeability change as outlined in (2), the membrane resistance would be decreased.

To determine which of the two alternative mechanisms (1) or (2) is responsible for the repolarization of the membrane and the neuromuscular block in the presence of persisting concentrations of acetylcholine determinations were made of the transverse membrane resistance at the end-plate region before and after the application of acetylcholine.

Methods.

The experiments were performed on the isolated sartorius nerve-muscle preparation of the frog (Rana temporaria) during September and October, at a room temperature of about 21° C.

The nerve-muscle preparation was mounted and the end-plate region of single muscle fibres located as previously described (THESLEFF 1955).

Determinations of the transverse membrane resistance of single muscle fibres were made as described by FATT and KATZ (1951). Two microelectrodes were inserted 25—50 μ apart in the muscle fibre. One of the microelectrodes was used to pass current through the

membrane and the other to record resulting change of membrane potential. The current was an inward directed pulse through the membrane of about 70 msec. duration and of an intensity which caused the membrane potential to increase by about 40 mV. The current was delivered by a rectangular pulse generator free from earth, and it was ascertained that a period of sufficiently steady current flow and steady membrane potential preceded the break of the pulse. The transverse membrane resistance is defined as the resistance from a point inside the fibre to the outside medium.

The microelectrode measuring the membrane potential was connected through an Ag—AgCl electrode to a d. c. amplifier (frequency response 0—35,000 c/s) with a cathode follower (RCA 954) as input stage, as described by NASTUK and HODGKIN (1950). The grid current was less than 5×10^{-11} A. The current passing through the cell membrane was measured by recording the potential difference across a resistance of 200 kΩ with a battery-operated d. c. amplifier in which the input stage was without connection to earth. Current and membrane potential were simultaneously recorded with single sweep on a double beam cathode-ray oscilloscope. The error of the determinations was about $\pm$ 10 per cent.

Resistance determinations were made at the end-plate region and in the nerve-free pelvic part of single muscle fibres. At each point, one determination was made before the addition of neostigmine and acetylcholine, and one fifteen minutes after the addition of acetylcholine iodide in a concentration of 20 μg/ml (35 minutes after the addition of neostigmine bromide in a concentration of 1 μg/ml.

The Ringer's fluid had the following ionic composition expressed in m Mole/l: Na 115; K 2.1; Ca 1.8; all salts were used as chlorides. A fresh solution of neostigmine bromide (Leo) was prepared for each day's experiment. Solutions of acetylcholine iodide (Hoffmann La-Roche) were prepared immediately prior to use.

Results and Discussion.

As previously described (THESLEFF 1955) acetylcholine in a concentration of 5—20 μg/ml causes a neuromuscular block which persists despite the fact that the resting membrane potential at the end-plate region is repolarized within 10—15 minutes. It is seen from Table 1 that the transverse membrane resistance at the end-plate region was normal fifteen minutes after the addition of acetylcholine in a concentration of 20 μg/ml. If, however, determinations were made shortly after the addition of acetylcholine, *i. e.* during the phase of repolarization, the transverse membrane resistance was reduced, and was not normalized until the resting membrane potential was almost repolarized.

Table 1.

The transverse muscle membrane resistance at the end-plate region before and fifteen minutes after the addition to the muscle bath of acetylcholine iodide 20 μg/ml in the presence of neostigmine bromide 1 μg/ml.

Fibre	Before		After	
	Resting membrane potential (mV)	Transverse membrane resistance (kΩ)	Resting membrane potential (mV)	Transverse membrane resistance (kΩ)
1	— 102	143	— 85	150
2	— 103	204	— 91	197
3	— 101	143	— 91	150
4	— 88	167	— 76	171
5	— 96	152	— 81	194
6	— 103	131	— 97	146
7	— 104	135	— 84	104
8	— 96	126	— 81	111
9	— 103	241	— 84	195
Mean	— 99.6	160	— 85.6	158

To investigate if acetylcholine caused an increase in the ionic permeability in other parts of the muscle membrane resistance measurements were made at the nerve-free pelvic end of ten muscle fibres. The mean values for resting membrane potential and transverse membrane resistance before the addition of acetylcholine were 91 mV, 199 kΩ those fifteen minutes after addition of acetylcholine being 80 mV and 189 kΩ.

The results show that when acetylcholine has produced a neuromuscular block and the resting membrane potential at the end-plate region is repolarized, the transverse resistance of the muscle membrane is normal. This excludes the possibility that the repolarization of the membrane and the neuromuscular block in the presence of persisting concentrations of acetylcholine is due to a selectively maintained increase in the potassium and chloride permeability of the cell membrane.

The other known mode of action which could account for the repolarization of the membrane and the neuromuscular block in the presence of acetylcholine was that exerted by tubocurarine. Tubocurarine is known to decrease the sensitivity of the end-plate region to the transmitter substance without altering the ionic permeability of the cell membrane, and it is this action which has been shown in the present investigation to be exerted by acetyl-

choline. *It can therefore be concluded that persisting high concentrations of acetylcholine block neuromuscular transmission by a mechanism similar to that of tubocurarine.*

Summary.

The repolarization of the end-plate region and the neuromuscular block caused in frog skeletal muscle by persisting high concentrations of acetylcholine could be due either to a mechanism reducing the reactivity of the end-plate "receptors" without affecting the membrane, *i. e.* a tubocurarine-like effect, or to the possibility that the membrane at the end-plate region was left highly permeable to ions other than sodium.

To determine which of these two alternative mechanisms is responsible for the effects of persisting concentrations of acetylcholine, determinations were made of the transverse electric membrane resistance at the end-plate region before and after the application of acetylcholine.

It was shown that the transverse membrane resistance was completely normal when acetylcholine had caused a neuromuscular block and the end-plate region was repolarized.

This excludes the possibility that the repolarization of the end-plate region with the neuromuscular block in the presence of persisting concentrations of acetylcholine is due to an increase in the ionic permeability of the muscle membrane. It is concluded that the only other known mode of action which could account for these effects of acetylcholine is that exerted by tubocurarine.

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The Effect of Anesthetic Agents on Skeletal Muscle Membrane.

By

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Anesthetic agents are known to depress the direct excitability of skeletal muscle, and in many instances to inhibit neuromuscular transmission (OVERTON 1902, LILLIE 1923, SECHER 1951, QUILLAM 1955, and others). This depressant effect on the excitability of the cell membrane could be explained by a reduction of the selective sodium conductance change in the active membrane. To investigate such a possibility, a study was made of the effects on the resting and the active skeletal muscle membrane of a number of potent and chemically different anesthetics.

The investigation was carried out on the isolated sartorius muscle preparation of the frog with the intracellular microelectrode technique, and the results were analyzed in accordance with the ionic hypothesis for the electric activity in nerve and muscle (HODGKIN and KATZ 1949). For technical reasons, only non-volatile anesthetics were used. The agents selected for the study were: pentobarbital sodium (USP), urethan, chloral hydrate, α-chloralose, paraldehyde and tribromethanol.

Of these agents, pentobarbital has a potent neuromuscular blocking action, as shown by QUILLAM (1955). This compound was therefore selected for a special study of its effects on the neuromuscular transmission in the frog.

Methods.

The experiments were performed on the isolated sartorius nerve-muscle preparation of the frog during December to May, at a room temperature of about 21° C. The effects of pentobarbital were investigated in Rana temporaria and Rana esculenta was used in the other experiments.

Recording of end-plate potentials was made by external recording, as previously described (FATT 1950, THESLEFF 1955).

Intracellular recording was made with conventional capillary micro-electrodes filled with 3M-KCl and having an external tip diameter of less than 0.5 μ. Only electrodes with a resistance of less than 10 MΩ were used.

The freshly dissected muscle was fastened on a transparent Perspex block in a Ringer bath. Illumination was provided by light passing up through the Perspex block, and the preparation was viewed with a binocular dissecting microscope. The microelectrode was connected to the input stage through an Ag-AgCl electrode, and the bath contained an agar-Ringer fluid bridge connecting to an Ag-AgCl electrode. The bath electrode led to earth via small series resistances, through which steady calibration voltages could be applied. The microelectrode assembly was carried on a Zeiss slide micromanipulator.

For recording of the resting and the active membrane potential, a d. c. amplifier was used (frequency response flat—40,000 c/s) with a cathode follower (RCA 954) as input stage, as described by NASTUK and HODGKIN 1950. The grid current was less than 5×10^{-11} A. The capacity of the input stage was of the order of 1 $\mu\mu$F.

Miniature end-plate potentials were recorded with a battery-operated a. c. preamplifier with a frequency response of 0.3—30,000 c/s.

The end-plate region of single muscle fibres was located by determining the point of maximum miniature end-plate potentials.

Acetylcholine was applied locally to single end-plate regions by electrophoretic release of acetylcholine ions from a micropipette, as described by DEL CASTILLO and KATZ (1955).

Measurements of the electric threshold of the muscle membrane were made in the nerve-free distal part of single muscle fibres by the use of two intracellular electrodes, as described by FATT and KATZ (1951). The duration of the stimulating current pulse was 50 msec., and the potential change from the resting level at which an action potential was generated was taken as the threshold. The amplitude of the action potential was measured from the resting level.

The transverse electric resistance of the muscle membrane was determined as described by FATT and KATZ (1951). The arrangement for recording is shown in Fig. 1. Measurements were made at the distal nerve-free region of single muscle fibres before and fifteen minutes after addition of the anesthetic agent to the muscle bath. The transverse membrane resistance is defined as the resistance from

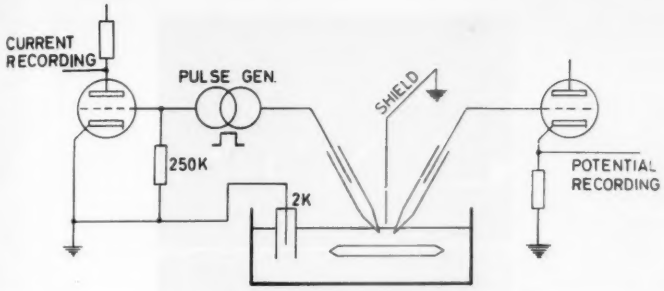

Fig. 1. Arrangement for measuring current and potential across the fibre membrane with two internal electrodes.

a point inside the fibre to the outside medium. The error of individual determinations was about $\pm$ 10 per cent.

The Ringer's fluid had the following ionic composition expressed in mMol/l: Na 114; K 2.1; Ca 1.8; Cl 109; HPO_4 4.0 and H_2PO_4 2.6. During the experiments the pH of the fluid remained within the limits of 6.6—7.4. The Ringer's fluid in the bath was changed about every 30 minutes in the course of the experiments.

The median hypnotic dose (HD^{50}) was determined in the frog by injection of the anesthetic agent into the ventral lymph sac, using at least ten frogs for each determination. The criterion of hypnosis was that the frog, when turned, should lie on its back for at least five minutes.

Results.

The effects of pentobarbital on neuromuscular transmission.

In a concentration of 0.20—0.30 mg/ml, pentobarbital sodium caused within 10—20 minutes a complete neuromuscular block in the isolated sartorius muscle of the frog. By washing the muscle with fresh Ringer's fluid the neuromuscular transmission was completely restored. With suitable concentrations of pentobarbital, end-plate potentials (e. p. p.) could be observed during the period of block and they were increased in size by repetitive stimulation of the motor nerve (Fig. 2).

To investigate the action of pentobarbital on the release of the transmitter by the motor-nerve terminals, the effect on the spontaneous miniature e. p. p.'s was studied.

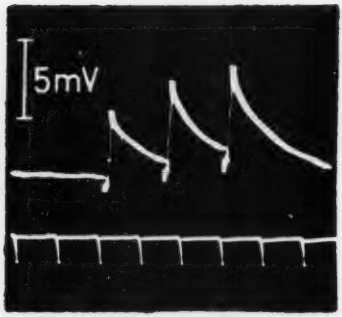

Fig. 2. The effect of repetitive motor-nerve stimulation on the end-plate potential during a neuromuscular block caused by pentobarbital sodium in a concentration of 0.3 mg/ml. Time 5 msec. (External recording.)

The present study confirmed the observations of FATT and KATZ (1952) that the miniature e. p. p.'s showed marked individual variations with respect to both size and frequency of discharge. The amplitude of the miniature e. p. p.'s ranged from 0.25 to 1 mV (Table 1) and great variations in their spontaneous frequency at different end-plate regions occurred (0.3—10.2 per sec.).

Pentobarbital in a concentration of 0.1 mg/ml reduced the amplitude of the miniature e. p. p.'s by about 50 per cent (Fig. 3). In higher concentrations they were completely abolished. The spontaneous discharge rate of the miniature e. p. p.'s was slightly reduced by pentobarbital in a concentration of 0.1 mg/ml (Table 1). The possibility nevertheless exists that this decrease in frequency was not due to a real reduction in the number of units of acetylcholine released, but merely to the fact that the diminution in size made it impossible to record the smallest miniature e. p. p.'s.

To investigate whether, in the presence of pentobarbital, the postsynaptic membrane was rendered insensitive to the depolarizing effect of the neuromuscular transmitter, acetylcholine was applied locally to the end-plate region of single muscle fibres by electrophoresis. The resting membrane potential of this region was measured simultaneously by an intracellular electrode. As shown in Fig. 4, pentobarbital in a concentration as low as 0.05 mg/ml markedly reduced the depolarization caused by acetyl-

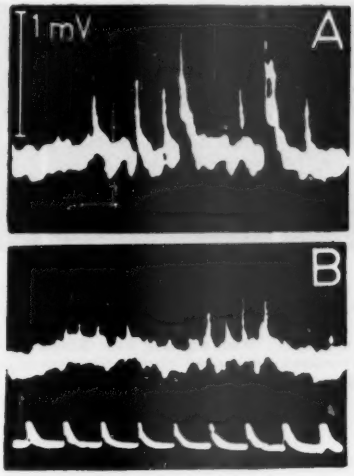

Fig. 3. Miniature end-plate potentials. A, before and B, after addition of pentobarbital sodium to the muscle bath in a concentration of 0.1 mg/ml. Time 0.1 sec.

Table 1.

Effect of pentobarbital sodium on frequency of spontaneous miniature end-plate potentials.

(In each experiment observations were made on the same end-plate before and after the addition of 0.1 mg/ml pentobarbital sodium to the muscle bath).

Expt	Discharges per sec.		Frequency ratio Normal/Pentobarbital
	Normal	After 0.1 mg/ml Pentobarbital	
1	3.4	2.1	1.62
2	0.3	0.3	1.00
3	4.6	3.4	1.35
4	0.5	0.4	1.25
5	0.5	0.5	1.00
6	7.8	6.3	1.24
7	7.9	7.3	1.08
8	8.7	7.6	1.14
9	7.7	8.6	0.90
10	10.2	6.5	1.57
11	6.7	5.3	1.26
Mean ± S. E. of mean			1.22 ± 0.690

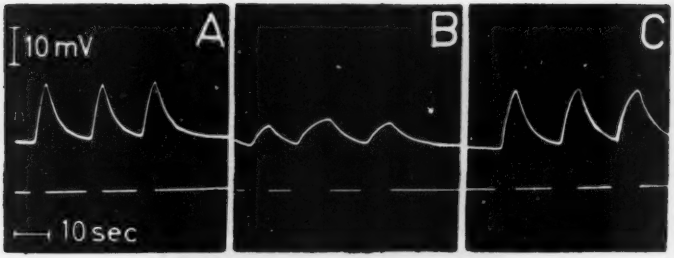

Fig. 4. The effect on resting membrane potential of acetylcholine released close to the end-plate region of a single muscle fibre by electrophoresis from a micropipette. A, before and B, after the addition of pentobarbital sodium in a concentration of 0.05 mg/ml. C, after washing with Ringer's fluid. The position of the micropipette remained unchanged during the experiment. The duration of the current passing through the micropipette is marked by the interruptions in the lower line. The resting membrane potential was 85 mV.

choline. In higher concentrations, pentobarbital completely abolished the depolarizing effect of acetylcholine.

The effect of anesthetic agents on skeletal muscle membrane.

The electric threshold of the membrane of single muscle fibres was determined with intracellular electrodes, the potential change from the resting level at which an action potential was generated being taken as the "threshold". It may be inferred from Table 2 that all anesthetics greatly increased the electric threshold of the membrane with the exception that pentobarbital in low concentrations significantly ($p < 0.01$) decreased it. It is also seen that the anesthetics caused a marked reduction in the amplitude of the action potential, and in higher concentrations completely abolished it in a large number of fibres.

It is evident from Table 2 that these effects on membrane excitability were not accompanied by any marked changes in the resting membrane potential.

Records were also made of propagated action potentials in muscle fibres before and after the addition of an anesthetic agent. As shown in Figs. 5 and 6, the anesthetics decreased the rate of rise of the action potential and, in addition to diminishing the amplitude, also lengthened the duration of the action potential.

Table 2.

The effect of anesthetic agents on the electric "threshold" of the muscle fibre membrane and on the amplitude of the resulting action potential.

Table 2.

The effect of anesthetic agents on the electric "threshold" of the muscle fibre membrane and on the amplitude of the resulting action potential.

Concentration of anesthetic agent (g/ml)	No. of muscles	Total no. of fibres	Mean resting membrane potential (mV)	No. of fibres with action potential	Mean "threshold" (mV)	Mean amplitude of action potential (mV)
Normal Ringer's fluid	4	63	94 ± [1]0.7	63	40 ± [1]0.5	126 ± [1]2.2
	[2]3	60	87 ± 0.9	60	37 ± 0.7	125 ± 1.2
Pentobarbital sodium						
1.5×10^{-4}	[2]2	27	80 ± 0.9	18	32 ± 1.3 $p < 0.01$	98 ± 3.3
2×10^{-4}	[2]3	35	79 ± 1.2	15	31 ± 1.7 $p < 0.01$	98 ± 3.8
3×10^{-4}	[2]2	22	81 ± 1.2	8	45 ± 1.3	90 ± 5.4
Urethan						
5×10^{-3}	3	37	93 ± 1.2	33	52 ± 1.1	123 ± 1.5
7.5×10^{-3}	2	47	98 ± 0.7	45	53 ± 1.2	117 ± 1.8
Chloral hydrate						
7.5×10^{-4}	2	40	92 ± 0.8	40	43 ± 0.6	116 ± 1.3
1.5×10^{-3}	2	44	93 ± 0.8	40	49 ± 1.3	100 ± 2.5
Chloralose						
1.5×10^{-3}	2	33	92 ± 1.0	33	49 ± 1.0	106 ± 2.0
2.5×10^{-3}	2	41	90 ± 0.7	26	49 ± 1.1	86 ± 3.5
Paraldehyde						
8×10^{-3}	2	27	89 ± 1.4	27	39 ± 1.1	115 ± 2.1
10^{-2}	2	36	97 ± 0.7	27	51 ± 1.6	100 ± 3.2
Tribromethanol						
5×10^{-4}	2	44	94 ± 0.9	43	52 ± 1.1	108 ± 2.1
10^{-3}	2	41	88 ± 0.8	16	55 ± 2.3	99 ± 5.2

[1] S. E. of mean. — [2] Rana temporaria.

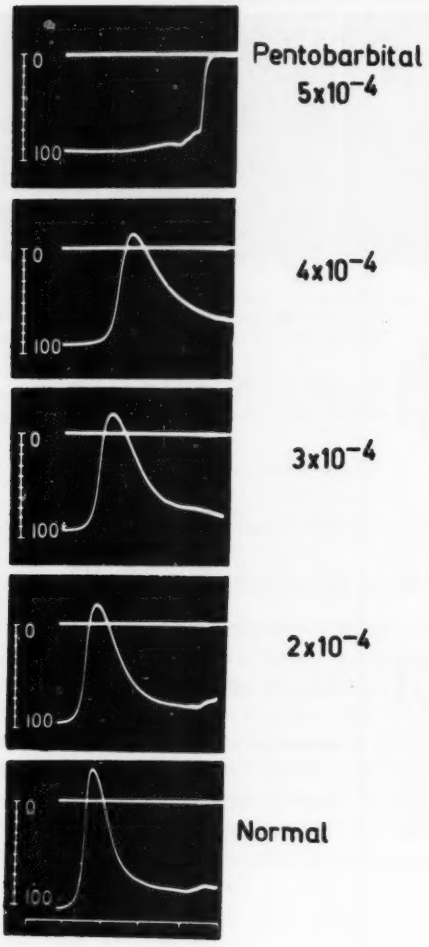

Fig. 5. The effect of increasing concentrations of pentobarbital sodium on the action potential of a single muscle fibre. Time in msec.

For an analysis of these effects it was necessary to determine whether the ionic permeability of the resting muscle membrane was increased by the anesthetics used. This was done by determinations of the transverse electric resistance of the fibre mem-

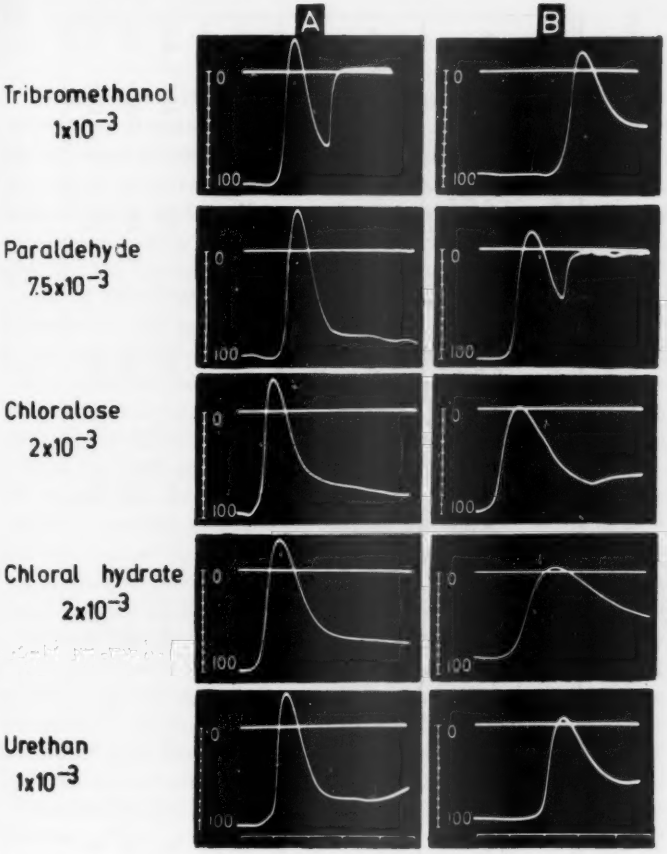

Fig. 6. The action potential of single muscle fibres. A, before and B, after addition of an anesthetic agent to the muscle bath. Time in msec.

brane before and after the addition of an anesthetic agent. From the results shown in Table 3 it is seen that none of the agents caused any considerable alteration in the transverse membrane resistance. Pentobarbital, chloralose and paraldehyde increased the resistance by about 20 per cent, while urethan and tribromethanol decreased it by about 15 per cent.

Table 3.

The transverse muscle membrane resistance at the distal nerve-free part of single muscle fibres before and fifteen minutes after the addition of an anesthetic agent to the muscle bath.

Concentration of anesthetic agent (g/ml)	No. of muscles	No. of fibres	In Ringer's fluid		After addition of anesthetic agent		Mean alteration in resistance (per cent)
			Mean resting membrane potential (mV)	Mean transverse membrane resistance (kΩ)	Mean resting membrane potential (mV)	Mean transverse membrane resistance (kΩ)	
Control	4	11	96.9	285	93.2	273	$-4.2 \pm^{1} 1.88$
Pentobarbital sodium 10^{-4}	7	7	96.0	473	91.3	521	$+10.1 \pm 2.42$
3×10^{-4}	7	7	96.0	473	83.3	581	$+22.8 \pm 7.10$
Urethan 10^{-2}	2	8	96.5	272	94.0	233	-14.3 ± 2.61
Chloral hydrate 2×10^{-3}	2	7	93.6	359	92.7	362	$+0.8 \pm 1.58$
Chloralose 3×10^{-3}	3	8	94.5	333	93.0	395	$+18.6 \pm 4.67$
Paraldehyde 10^{-2}	2	8	92.0	345	88.3	416	$+20.6 \pm 2.92$
Tribromethanol 10^{-3}	3	8	92.4	341	90.5	290	-15.0 ± 2.38

[1] S. E. of mean.

Discussion.

According to the ionic hypothesis of HODGKIN and KATZ (1949) and HODGKIN (1951), the action potential in nerve and skeletal muscle fibre is the result of a high, selective and brief increase in the permeability of the cell membrane to sodium ions, followed after a delay by a prolonged increase in permeability to potassium ions. The increased sodium conductance is responsible for the rising phase of the action potential and for the reversal of the membrane potential. The subsequent increase in permeability to potassium ions counteracts the depolarization and is responsible for the rapid repolarization of the membrane.

The selective transport of sodium ions across the membrane is considered to be due to the activation of a hypothetical sodium carrying mechanism in the cell membrane. The rate of change in sodium conductance is determined by the membrane potential. The magnitude of the sodium current is a function of the "sodium carrying mechanism" and of the sodium concentration gradient across the membrane.

The electric threshold of the membrane is the level of depolarization at which the sodium conductance exceeds that of potassium ions. The amplitude of the action potential is determined by the selective and rapid sodium conductance change, and is diminished by an increased potassium conductance. The electric threshold of the membrane and the amplitude of the action potential are thus determined by the relationship between the conductance of sodium ions and that of potassium.

The membrane resistance is inversely proportional to the conductance of the membrane which, in the resting state, is mainly due to the potassium permeability (HODGKIN).

In the present investigation it has been shown that anesthetic agents increase the electric threshold of the muscle membrane and reduce or abolish the action potential. These effects were brought about without a significant change in the resting membrane potential, and the concentration gradient for sodium was not altered during the experiments. The transverse resistance of the resting membrane was generally not reduced and consequently, neither was the resting potassium conductance increased. *The most reasonable explanation to the above findings seems to be that the sodium current in the active muscle membrane is reduced by the*

anesthetic agents, and that this effect is caused by a reduction of the available "sodium carrying mechanism". Whether the drugs have a similar effect or not on the potassium conductance change has not been investigated.

This explanation can account for the effects brought about by all the agents used in this study as well as those produced by low concentrations of pentobarbital. In a concentration of 0.15—0.20 mg/ml, pentobarbital caused a significant decrease in the electric threshold of the membrane and at the same time reduced or abolished the action potential. A possible explanation of these effects is that pentobarbital, besides reducing in the active membrane the sodium conductance change, also diminished the potassium conductance in the resting membrane. That this is a likely explanation is suggested by the observation that pentobarbital consistently caused an increase in the transverse resistance of the resting muscle membrane (Table 3).

The rate of rise of an action potential is directly proportional to the rate of entrance of sodium ions, *i. e.* to the selective sodium conductance (HODGKIN and KATZ). In the present experiments, the anesthetic agents caused a decrease in the rate of rise of the action potential (Figs. 5 and 6) and thereby provided further evidence for the statement that they diminish the selective sodium conductance change ("sodium carrying mechanism") in the muscle fibre membrane.

It was also observed that pentobarbital markedly reduced the sensitivity of the end-plate to applied acetylcholine. As shown by FATT and KATZ (1951), it is unlikely that depolarization of the end-plate region of the muscle membrane is caused by a selective change in sodium conductance. The unreactivity of the end-plate region cannot therefore be explained by the observed inhibition of this mechanism. A possible explanation is that pentobarbital has rendered the end-plate "receptors" unreactive to the depolarizing effect of acetylcholine, *i. e.* has a mode of action similar to that of tubocurarine.

Not only excitation of nerve fibre and skeletal muscle, but also excitation of heart muscle and of motoneurons is accompanied by a selective increase in the sodium conductance in the cell membrane, as shown by HODGKIN and KATZ, NASTUK and HODGKIN, DRAPER and WEIDMANN (1951) and COOMBS, ECCLES and FATT (1955). It is known that anesthetic agents depress or abolish the excitability of all these structures. Whether excita-

Table 4.

Comparison between the hypnotic dose in the intact frog and the concentration which, in the isolated sartorius muscle preparation, caused a reduction in the selective sodium conductance change in the muscle membrane.

Anesthetic agent	A Mean hypnotic dose (mg/g body weight)	B Concentration reducing the selective sodium conductance in muscle membrane (mg/ml)	Ratio A/B
Pentobarbital sodium	0.03	0.15	0.20
Urethan	2.5	5.0	0.50
Chloral hydrate.....	0.75	0.75	1.0
Chloralose	0.4	1.5	0.27
Paraldehyde	1.0	8.0	0.13
Tribromethanol	0.1	0.5	0.20

tion and impulse transmission in the central nervous system are caused by the same selective ionic conductances is not known, but such a possibility is considered highly probable (HODGKIN 1951, ECCLES 1953).

The hypnotic and anesthetic effect of an agent could thus be the result of an inhibitory action on a selective sodium conductance change in the neurons of the central nervous system. If this occurred, a correlation could be expected between the hypnotic concentration and the concentration causing a reduction of the selective sodium conductance change in the muscle membrane. To investigate this possibility, the mean hypnotic dose in the frog was determined for each compound, and the ratio between the mean hypnotic dose (mg/g body weight) and the concentration (mg/ml) reducing the selective sodium conductance change in the frog skeletal muscle was calculated. The results are shown in Table 4. Despite a 100-fold difference between the effective dose of the various agents, the ratio between the hypnotic dose and the concentration affecting the selective sodium conductance change in the muscle membrane was observed to be remarkably constant.

A number of widely differing chemical agents produced in the present investigation both an anesthetic action and an inhibitory effect on the selective sodium conductance change in the skeletal

muscle membrane. Furthermore a correlation seemed to exist between the hypnotic action of these agents and their inhibitory effect on the selective sodium conductance change. *It is therefore suggested that the hypnotic and anesthetic activity of these drugs is due to a reduction of a selective sodium conductance change in the active cell membrane of certain neurons in the central nervous system and that this effect is caused by an inhibition of the "sodium carrying mechanism".*

Summary.

The action of pentobarbital sodium on neuromuscular transmission and the effects of pentobarbital, urethan, chloral hydrate, chloralose, paraldehyde and tribromethanol on skeletal muscle excitability has been investigated with the intracellular microelectrode technique in the isolated sartorius nerve-muscle preparation of the frog.

Pentobarbital sodium blocked neuromuscular transmission by rendering the end-plate "receptors" unreactive to the depolarizing effect of acetylcholine.

All agents caused an increase in the electric "threshold" of the muscle membrane and reduced or abolished the action potential. The rate of rise of an action potential was decreased by all agents.

These effects occurred without significant changes in the resting membrane potential of the muscle fibre and without a decrease in the transverse electric resistance of the muscle membrane.

On the basis of the ionic hypothesis for electric activity in nerve and muscle (HODGKIN and KATZ 1949), it is suggested that these effects on the membrane "threshold" and on the action potential are due to a reduction of the selective sodium conductance change caused by an inactivation of the "sodium carrying mechanism" in the muscle membrane.

A close relationship was observed between the hypnotic dose in the intact frog and the concentration of the respective agent that caused a reduction of the selective sodium conductance change in the frog skeletal muscle membrane.

It is suggested that the hypnotic and anesthetic activity of agents may be due to a reduction of the selective sodium conductance change in the cell membrane of certain neurons in the central

nervous system and that this effect is caused by an inhibition of a "sodium carrying mechanism".

Acknowledgement: Technical assistance was given by Miss E. ADLER.

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Histamine and Mast Cells.[1]

Studies on Living Connective Tissue in the Hamster Cheek Pouch.

By

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Received 12 June 1956.

Mast cells are generally believed to be the origin of the acid mucopolysaccharides heparin (JORPES et al. 1937) and hyaluronic acid (ASBOE-HANSEN 1950).

In 1953 RILEY and WEST advanced the theory that the mast cells contain histamine which can be released to the tissues. Since then several authors have stressed the parallelism between the mast-cell content of a tissue and its histamine value (RILEY and WEST 1953, 1955, CASS, RILEY, WEST, HEAD and STROUD 1954).

GRAHAM, LOWRY, WHEELWRIGHT, LENZ and PARISH (1955) noted a considerably higher histamine content in the basophil leukocytes ("blood mast cells") than in other blood cells. There is some evidence that the basophil leukocyte and the tissue mast cell are similar in type (EHRICH 1953, ASBOE-HANSEN and KAALUND-JØRGENSEN 1956).

In rats RILEY (1953) observed disruption and degranulation of the tissue mast cells in the mesentery following intraperitoneal as well as intravenous injection of chemical histamine-liberators.

[1] Aided by grants from Eli Lilly and Co., Indianapolis, Indiana, U. S. A., the National Danish Association against Rheumatic Diseases, the Danish Anti-Cancer League, and Merchant in Odense Johann and Hanne Weimann, née Seedorff's Foundation.

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These changes were prevented by premedication with antihistamines. In areas of histamine-induced tissue oedema mast-cell degranulation was also observed, but these changes were considered different in quantity and quality. In 1955 RILEY, SHEPHERD, WEST and STROUD could not find conformity between the liberation of histamine and heparin in rats, though disruption and degranulation of the mast cells occurred in response to injection of the chemical histamine-liberator substance 48/80.

Intravenous injection of peptone is reported to induce histamine shock and incoagulability of the blood in the dog (KELLAWAY 1947), a parallelism which is not found in other species (ADAMS 1953).

Increased histamine content with simultaneous disruption of the mast cells has been observed by BENDITT, BADER, ARASE, CORLEY and LAM (1954) in peripheral oedema induced with ovomucoid in rats.

The mentioned morphological changes have been observed in *fixed* material.

There is much evidence that at least some mast cells contain considerable amounts of histamine; but the problem whether the mast-cell changes are a specific response to the histamine-liberators, in some way bound up with the liberation of histamine, or a response to the tissue *effects* of the histamine liberated from other sources, is still unsolved.

The aim of this work was to study — under conditions as physiological as possible — the responses of *living* mast cells to histamine as well as to histamine-liberators in connective tissue receiving unhindered blood supply and on the whole left largely intact.

Material and Methods.

Mast cells were examined in the connective tissue of the cheek pouch of the hamster (*Mesocricetus auratus*) under nembutal anæsthesia by a method previously described (WEGELIUS and HJELMMAN 1955; WEGELIUS and ASBOE-HANSEN 1956). The tissues were studied in the bright field as well as in the phase contrast microscope by means of water immersion objectives.

The effects of a neutral and isotonic solution of histamine hydrochloride, of compound 48/80 in physiological saline solution, of stilbamidine isethionate (M. & B.) in sterile water, of peptone (Witte) in physiological saline, and of sterile water were tested. Stilbamidine is highly toxic; the intracardiac injections were given slowly in order

Table 1.

Influence of histamine and histamine-liberator substances on living tissue mast cells. Agents administered by intracardiac, intramuscular, and intraperitoneal injections. The observation periods were 3, 15, 30, 40 and 60 min.

Agent	Conc.	Dose	Mast cell response
Histamine hchl.	1 mg/1 ml	0.5—2 mg	Degranul. Clumping
Cp. 48/80	1 mg/1 ml	0.4 mg	Degranul. Clumping
Stilbamidine isethionate	10 mg/1 ml	7.5 mg	Degranul. Clumping
Peptone	100 mg/1 ml	50—100 mg	Degranul. Clumping
Sterile water		2 ml	Degranul. Clumping Disruption

to avoid immediate death. Compound 48/80, a polymeric condensation product of p-methoxyphenethyl-methylamine and formaldehyde[1], is a more specific histamine-liberator of lower toxicity (PATON 1951, RILEY and WEST 1955). Water induces an osmotic imbalance and is believed to liberate histamine locally (FAWCETT 1954).

As a control the left cheek pouch was examined before the distant injections of the mentioned agents. The connective tissue of the divided cheek pouch was stained for 5 minutes with 1 ‰ toluidine blue in physiological saline. During this procedure the staining of the tissue was studied in the microscope, until the dye had diffused throughout the tissue. Thereafter shock doses of the agents were injected intramuscularly, intraperitoneally, or intracardially. The concentrations of the solutions are listed in table 1. The effects were studied in the right cheek pouch, handled and stained in exactly the same way as the control pouch.

For *control* purposes physiological saline and Tyrode solution were administered in the same amounts by distant injections to a series of animals.

Part of the investigations aimed at observing the very course of the mast-cell changes under the microscope. Using the *phase contrast microscope* relatively accurate observation of the mast-cell structure was possible even without staining. Thus, any action of the dye upon the metabolism and life of the cells was eliminated. When one or more

[1] Kindly supplied by Burroughs Wellcome & Co., England.

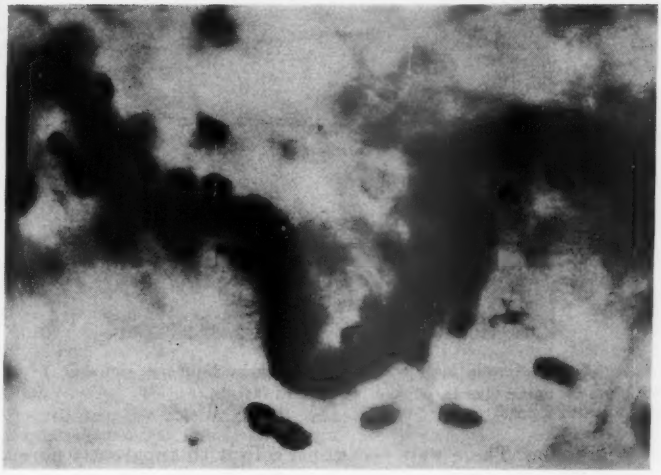

Fig. 1. Normal living mast cells along an arteriole in a cheek pouch of a hamster. Staining: toluidine blue in physiol. saline 1 : 1,000. Magnification: 500 ×.

mast cells with distinct granular structure had been visualized in a field, the different agents were injected, and the reactions of the cells were observed.

Results.

A few minutes after the injections the animals showed signs of shock, erythema, cyanosis and oedema of the muzzle and the paws. The connective tissue of the cheek pouches looked more or less oedematous.

Distinct, and often extremely marked, degranulation of the mast cells was observed following systemic administration of shock doses of histamine as well as the histamine-liberators 48/80 and stilbamidine (Fig. 3). The effect on the cells of compound 48/80 was observed to set in within 5 minutes after the injection, and to reach a maximum within 30 minutes. Peptone shock induces slight degranulation, but never as marked as the above-mentioned agents. Following systemic administration of sterile water some cells were irreversibly disrupted (Fig. 4), but most cells remained intact, though degranulated. Clumping of the granules was noticed in some of those cells which still retained

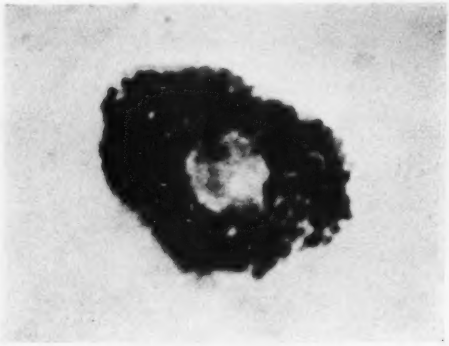

Fig. 2. Normal mast cell loaded with metachromatic granules.
Staining: toluidine blue in physiol. saline 1 : 1,000.
Magnification: 2,200 ×.

their granules. There were occasional cells with apparently normal granulation (Fig. 2) or even groups of such cells, although almost exclusively around the larger blood vessels. By contrast, pronounced degranulation was always observed around the capillaries, small arterioles, and venules.

In the control experiments involving systemic administration of physiological saline and Tyrode solution similarly applied, and in the same amounts, degranulation was not more marked than in the directly stained specimens (Fig. 1).

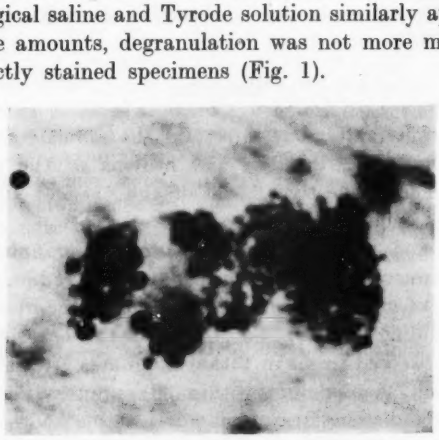

Fig. 3. Mast cell showing partial degranulation after intraperitoneal injection of 2 mg of histamine hydrochloride in isotonic solution. Notice the basic structure simulating a sponge or a honeycomb.
Staining: toluidine blue in physiol. saline 1 : 1,000.
Magnification: 2,200 ×.

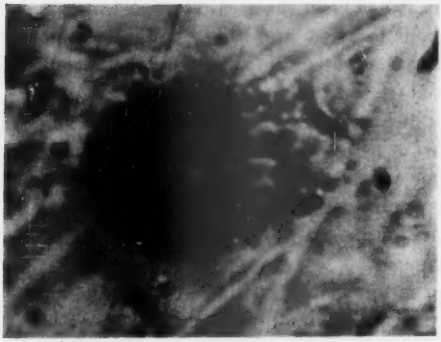

Fig. 4. Mast cell showing disruption after intraperitoneal injection of sterile water, 2 ml.
Staining: toluidine blue in physiol. saline 1 : 1,000.
Magnification: 2,200 ×.

When studying mast cells visualized in the phase contrast microscope without staining, the authors were able to observe changes in the mast cells after injection of histamine and histamine-liberators. The cell contours became indistinct, and the granular structure was altered; rearrangement of the granules and vacuolation were observed; sometimes the pattern was entirely blurred. Apparently unaffected mast cells were also nearly always demonstrable by this method.

It is obvious that two essentially different phenomena occurred in all experiments. A *degranulation, i. e.* a loss of granules, occasionally preceded by a granule clumping or confluence, is the common mast-cell response to the administration of the mentioned agents. To a slight extent, however, a true *disruption, i. e.* an irreversible break-down of the cells, is observed even in the controls; this may be due to the manipulations and the inevitable mechanical traumatization of the cheek pouches. The extent and velocity of the changes depended on the dosage.

Discussion.

The degranulation of the mast cells invariably seen after administration of histamine and histamine-liberators shows that the mast cells are sensitive to the influence of both.

Previously it has been shown that even water and physiological saline applied locally, and even traumatization may induce morphological changes similar to these (FAWCETT 1955, WEGELIUS and ASBOE-HANSEN 1956).

The doses of histamine and histamine-liberators are sufficiently large to start a series of pathophysiological processes. One common outcome of the different influences is, however, the tissue oedema. Histamine increases capillary permeability and histamine-liberators are supposed to cause a tissue oedema of a similar kind and genesis.

The observation that histamine exerts an action as powerful as that of the histamine-liberators indicates that the mast-cell changes are an unspecific response to the *effect of* histamine on tissues rather than a specific response to histamine-liberators.

When disintegration of the cell occurs, the contents are released; consequently, if the mast cells do contain histamine, this substance is also released. Whether degranulation, which is a function of the *living* cell, entails a release of histamine still remains an open question. It seems probable that the degranulation of the mast cells observed is a response to increased tissue fluid, which may be caused by histamine, directly or via histamine-liberator substances. This view is supported by the finding that the degranulation of the mast cells takes place principally and primarily around the capillaries and the smallest vessels, and to a lesser degree around the larger vessels.

There is much evidence that the mast-cell granules contain precursors of heparin and hyaluronic acid, and that these acid mucopolysaccharides may be released as a response to various influences (ASBOE-HANSEN 1950—1954). Hyaluronic acid is an important ground-substance component known to be involved in the binding of water (MEYER 1947).

The mast-cell degranulation observed in these experiments may be interpreted as a compensating endeavour to maintain the gel structure of the intercellular matrix by releasing water-binding mucinous substances of the mucopolysaccharide type.

The conclusion, based on experiments with *living* tissue and cells, assigns to the mast cell an acceptable position in relation both to histamine and tissue mucopolysaccharides.

Summary.

The reaction of the mast cells to histamine, the chemical histamine-liberators compound 48/80 and stilbamidine, peptone and water was studied in *living* connective tissue of the hamster cheek pouch. Degranulation of the cells was invariably observed and occasionally irreversible disruption. The degranulation is interpreted as a response to increased tissue fluid, which in turn may be due to increased capillary permeability. The mucopolysaccharide-containing granular substance of the mast cells may bind extracellular water and thus maintain the gel structure of the connective-tissue ground substance. This view unites the results of previous experiments on the relation of the mast cells to histamine as well as to tissue mucopolysaccharides.

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Influence of Cholesterol Feeding on the Distribution of Polyenoic Fatty Acids in the Liver of Chicks.

By

HENRIK DAM and GUNHILD KOFOED NIELSEN.

Received 18 June 1956.

In a previous communication (DAM, KRISTENSEN, NIELSEN, PRANGE and SØNDERGAARD 1956) we have described the influence of varied levels of peanut oil and cholesterol on cholesterol and polyenoic fatty acids in tissues of chicks.

With dietary peanut oil levels ranging from 0 to 20 % and cholesterol values from 0 to 1 % the variations of cholesterol seemed to have no influence on the distribution of polyenoic fatty acids in the tissues examined except for the combination 20 % peanut oil + 1 % cholesterol which resulted in a lowering of tetraenoic acid in the liver expressed as per cent of the total fatty acids.

In order to be quite certain about this latter question we have now repeated the part of the experiment which concerns the influence on liver polyenoic acids of 0 and 1 % cholesterol given with fat-free diets and with diets containing 10 and 20 % of peanut oil.

Methods and plan of the experiment was as described in the above-mentioned paper.

The results are presented in tables 1—4.

From table 1 it is seen that with diets containing 10 % and especially with 20 % of peanut oil increase of cholesterol from 0 to 1 % of the diet has actually depressed the amount of tetraenoic acid in the liver measured as per cent of the total fatty acids.

Table 1.

Polyenoic acids in per cent of total fatty acids in livers of chicks fed diets with varying amounts of cholesterol and peanut oil for 4 weeks.

Group no.	1637[1]	1638[2]	1639[1]	1640[1]	1641[1]	1642[2]
Addition % cholesterol ...	0	1	0	1	0	1
to diet % peanut oil ...	0	0	10	10	20	20
% dienoic	1.1	1.4	10.8	13.1	16.5	15.8
% trienoic	3.9	4.2	1.9	2.1	1.7	1.3
% tetraenoic	1.8	1.8	13.1	8.1	15.4	7.0
% pentaenoic	0.2	0.1	0.9	0.3	0.9	0.2
% hexaenoic	0.6	0.5	1.1	0.8	0.9	0.4
% polyenoic (total)	7.6	8.0	27.8	24.4	35.4	24.7

[1] Each group consisted of 8 animals. Two organs were pooled for each analysis. The figures listed represent averages of four such analyses.
[2] Each group consisted of 6 animals. Two organs were pooled for each analysis. The figures listed represent averages of three such analyses.

Table 2.

Content of polyenoic and total fatty acids expressed as mg per g liver (average of each group).

Group no.	1637	1638	1639	1640	1641	1642
Addition % cholesterol ...	0	1	0	1	0	1
to diet % peanut oil ...	0	0	10	10	20	20
dienoic	0.6	0.7	3.6	5.6	4.9	7.7
trienoic	1.8	1.8	0.6	0.9	0.5	0.6
tetraenoic	0.8	0.8	4.2	3.5	4.5	3.5
pentaenoic	0.1	0	0.3	0.1	0.3	0.1
hexaenoic	0.3	0.3	0.4	0.3	0.3	0.2
polyenoic (total)	3.6	3.6	9.1	10.4	10.5	12.1
total fatty acids	49.9	64.7	34.7	42.5	29.5	50.3

The small amounts of penta- and hexaenoic acids seem to have been influenced in the same direction, especially the pentaenoic acids.

Dienoic and trienoic acids were not influenced significantly. However, when expressed as mg per g liver (table 2) dienoic increased somewhat, when 1 % of cholesterol was fed with 10 or 20 % peanut oil. The increase of dienoic was more marked when expressed as mg per whole liver (table 4).

Table 3.

Average weight of livers in the groups.

Group no.	1637	1638	1639	1640	1641	1642
Weight of livers, g	6.4	6.2	8.2	8.2	6.0	7.3

Table 4.

Content of polyenoic acids as mg per liver (average of each group).

Group no.	1637	1638	1639	1640	1641	1642
Addition % cholesterol ...	0	1	0	1	0	1
to diet % peanut oil ...	0	0	10	10	20	20
dienoic	3.8	4.3	29.5	45.9	29.4	56.2
trienoic	11.5	11.2	4.9	7.4	3.0	4.4
tetraenoic	5.1	5.0	34.4	28.7	27.0	25.6
pentaenoic	0.6	0	2.5	0.8	1.8	0.7
hexaenoic	1.9	1.9	3.3	2.5	1.8	1.5
polyenoic (total)	22.9	22.4	74.6	85.3	63.0	88.4

With fat-free diets the distribution of polyenoic acids in the liver seemed to be the same, whether the diet contains 0 or 1 % of cholesterol.

These findings are in agreement with the suggestion of a relation between cholesterol and polyenoic acids set forth by other investigators (cf. DEUEL 1955).

The reduction of the amount of tetraenoic acid might be the result of an increased catabolism of this acid in the liver caused by the presence of a high amount of cholesterol deposited in this tissue (cf. DAM, KRISTENSEN, NIELSEN, PRANGE and SØNDERGAARD 1956). Another possibility might be that cholesterol by combining with linoleic acid to form cholesterol linoleate would interfere with the conversion of dienoic to tetraenoic acid. The fact that the amount of dienoic acid increases when expressed per g liver or per whole liver accentuates the decrease in tetraenoic expressed as per cent of total fatty acids.

Further experimentation is required to decide finally on the explanation of the results.

Summary.

Feeding of cholesterol at a dietary level of 1 % together with 10 or 20 % peanut oil to chicks caused a decrease in the amount

of tetraenoic acid in the liver, especially when expressed as per cent of total fatty acids.

When the diet did not contain fat, 1 % cholesterol had no influence on the amount of liver tetraenoic acid.

Liver pentaenoic acid and hexaenoic acids, the amounts of which were low under the conditions of the experiment, seemed to be influenced in the same direction as tetraenoic.

No significant change in liver dienoic or trienoic acids expressed as per cent of total fatty acids was found as a consequence of feeding 1 % cholesterol. When expressed as mg per g liver or per whole liver dienoic increased when 1 % cholesterol was fed with 10 or 20 % peanut oil.

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Quantitative Aspects of the Sympathetic Neuro-hormonal Control of the Heart Rate.

By

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It is evident from a recent study (CELANDER 1954) that the direct constrictor fibre control of the blood vessels is by far superior to the constrictor effects which can be induced by the hormones from the adrenal medulla, when activated at similar stimulation rates. On the other hand it is known that even a moderate secretion from this gland can induce powerful effects on specific metabolic events *e. g.* in skeletal muscle and liver cells, which lack a direct sympathetic innervation, indicating a clear-cut functional differentiation between the two links of the sympatho-adrenal system (see *e. g.* EULER 1953 and CELANDER 1953, 1954).

It is often taken more or less for granted, however, that the hormones from the adrenal medulla are of considerable importance for the control of the heart activity under normal circumstances, as even small, 'physiological' amounts of adrenaline can exert significant effects (see *e. g.* KJELLBERG et al. 1951). The relative importance of the hormonal link of the sympatho-adrenal system can, however, only be correctly judged when related to the range of control that is exercised by the cardioaccelerans nerves. — To allow such a comparison the direct sympathetic innervation of the heart and the adrenal medullas have been activated at maximal stimulation strengths at known frequencies, using the changes of heart rate as an indicator of the effector response.

Methods.

Experiments have been performed on ten cats, anaesthetized with 50 mg chloralose and 100 mg urethan per kg of bodyweight. Artificial respiration was given and so adapted that spontaneous respiratory movements were just suppressed. The thorax was then opened by dividing the sternum at its midpoint with extirpation of the second and third ribs down to the site of the sympathetic chains, so that the structures in the upper part of the thorax cavity could easily be exposed completely. The thoracic parts of the two sympathetic ganglionic chains were dissected free and all visible branches running towards the heart were carefully freed so that they all could be placed on electrodes for a concomitant stimulation. Other connections of the sympathetic chains were cut in order to disconnect the heart from the influence of central sympathetic structures. Also the two vagal nerves were divided and their peripheral ends dissected free to allow application of electrodes.

The upper part of the abdominal cavity was opened and the jejunum, ileum and colon were extirpated, leaving the blood supply to the stomach, the duodenum and the liver intact. The right adrenal gland was extirpated and the two splanchnic nerves of the left side were carefully dissected free and centrally cut so that their peripheral ends could be stimulated. — All the isolated nerve trunks were kept moist by cotton, soaked in oxygenated blood, and the exposed tissues were protected against drying and cooling by a thin plastic cover and a heat lamp.

This preparation thus allowed a stimulation of practically all the sympathetic fibres running to the heart and the nerves controlling the left adrenal medulla. As a parameter of the effector response to stimulation, the heart rate was continuously measured by way of a pulse recorder, operating an ordinate writer. Also the blood pressure was recorded with a mercury manometer connected to one of the femoral arteries. Electrical stimuli were delivered by Grass, model S4 C, stimulators, utilizing 5 millisecond square wave pulses at frequencies from one impulse every other second to forty impulses per second. The stimulation strength was in each case increased to such a level that a maximal effect for a given stimulation rate was reached to ascertain that the total number of fibres were activated. The stimulation periods varied between 15 to 90 seconds to allow the effector responses to become fully established. To compensate for the fact that only one of the two adrenal glands were activated, while — as far as was technically possible — all the cardioaccelerans fibres were stimulated, the effector response to a given accelerans fibre stimulation was throughout the experiments related to the effects of a left splanchnic stimulation at the double frequency. It was then assumed that the secretory capacity of the two adrenal medullas is approximately the same, which there are good reasons to believe (Celander 1954). Often the sympathetic stimulations were performed

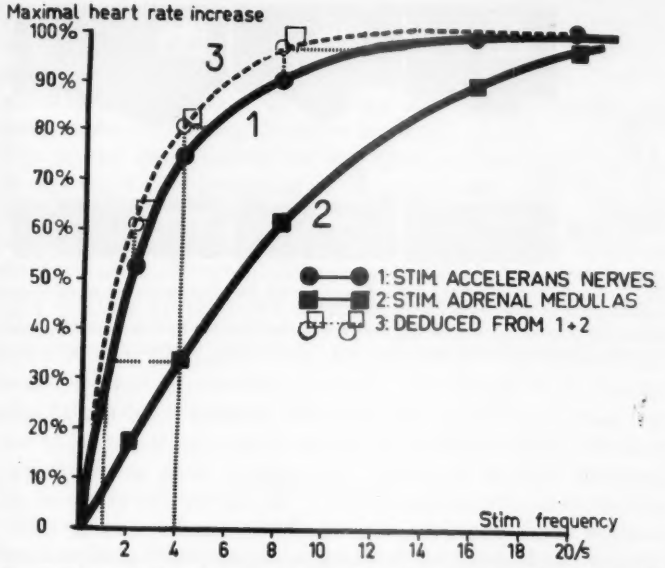

Fig. 1. The correlation between the stimulation frequency and the average (10 experiments) effector response to: Accelerans nerve stimulation (Curve 1). Activation of both adrenal medullas (Curve 2). A concomitant activation of both these links of the sympatho-adrenal system (Curve 3, deduced from Curves 1 and 2). For further explanation see the text.

against a background of a constant vagal stimulation at 4 or 6 impulses per second, a procedure which kept the heart rate low and remarkably constant as long as no sympathetic activation was induced.

Results.

As seen from Fig. 1, the correlation of the heart response to stimulation of the accelerans nerves at increasing rates is expressed by a hyperbolic curve (Curve 1), which is characteristic for most autonomic neuroeffectors (ROSENBLUETH 1950, CELANDER 1954, FOLKOW 1955). The effect on the heart rate is considerable already at very low stimulation rates, reaching about 90 per cent of the maximal obtainable level at 8 to 10 impulses per second, a correlation which, incidentally, was generally also seen at graded stimulations of the vagal nerves, confirming earlier studies (see ROSENBLUETH 1950). Curve 2 in Fig. 1 illustrates

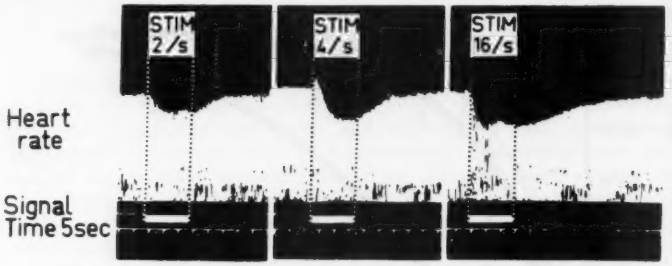

Fig. 2. An illustration of the rate of appearance and disappearance of the heart rate response to accelerans nerve stimulations at 2 impulses per second (1), 4 impulses per second (2) and 16 impulses per second (3). For further explanation see the text. The ordinates are approximately inversely proportional to the heart rate.

the heart response to the hormones released from the adrenal medullas, when activated at similar rates. Also the effect of the hormonal link is thus quite pronounced, when activated separately, but nevertheless definitely inferior to the effect of accelerans nerve stimulation, especially at the lower rates of excitation. At higher stimulation rates, corresponding to maximal activity levels of the sympatho-adrenal system (see FOLKOW 1955), also the hormonal link is able to bring about an almost maximal heart response. It must be remembered, however, that at intense sympathetic activation in the intact organism a maximal heart effect is already induced by the accelerans nerves and then the addition of the hormonal component makes little difference. It was also observed in some of the present experiments that a concomitant stimulation of both links at maximal rates did not materially increase the effector response as compared with the effect of accelerans nerve stimulation alone. — Curve 3 in Fig. 1 is meant to give an idea as to the effect on the heart rate of a generalized sympatho-adrenal activation in comparison with separate activation of the nervous and the hormonal links. It is seen from Curves 1 and 2 that the response to an activation of the adrenal medullas at *e. g.* 4 impulses per second is approximately the same as that of an accelerans nerve stimulation at 1 impulse per second. It seems reasonable to assume that the effect on the heart of a concomitant activation of both the accelerans nerves and the adrenal medullas in principle is an additive one, as the released ergones are closely related, if not identical. Therefore, a concomitant stimulation of the two links at 4 im-

pulses per second can be expected to induce an effect roughly corresponding to that of an accelerans stimulation at 5 impulses per second, etc. The small shifts of the responses from Curve 1 to Curve 3 indicate that the hormonal link only is of minor importance for controlling the heart activity.

As is also characteristic for the effects on the blood vessels of constrictor fibre stimulations (FOLKOW 1952), the heart response to low frequency stimulation of the accelerans nerves reached its maximum comparatively slowly. When interrupting the stimulation, however, the decrease of heart rate was sometimes obvious within a few seconds, reaching almost the basal rate within ten seconds in some experiments ('1' in Fig. 2). At higher rates of stimulation, on the other hand, the effector response reached its maximum rapidly, but lasted much longer after completed stimulation ('3' in Fig. 2).

Discussion.

The present experiments indicate that the direct sympathetic influence on the heart rate is by far superior to the effects that can be induced by the hormonal link of the sympathoadrenal system, which is in general agreement with quantitative studies of other autonomic effectors (CELANDER 1953, 1954). It is probable that this correlation also is applicable to the positive inotropic effect which is induced by the sympathetic innervation of the heart. ANZOLA and RUSHMER (1956) were recently able to show the remarkably strong effects which these nerves are able to induce on the contractility of the myocardium. In fact, the predominance of the accelerans nerves illustrated here, is probably even more pronounced — if anything — in the intact organism. The reason for this is that for technical reasons it is rather unlikely that the total number of accelerans fibres really have been prepared free and thus activated. On the other hand it is comparatively easy to isolate and stimulate at least the great majority of the nerve fibres running to the adrenal glands. Therefore the hormonal link may be somewhat favoured in the present experimental comparison.

It is obvious from Fig. 1 that the hormonal link, when activated alone, can induce intense effects on the heart, which explains why the adrenal medullary hormones often are looked upon as a most important sympathetic regulator of the heart. When,

however, these hormones affect the heart concomitantly with the accelerans nerves, as is normally the case, their relative influence becomes quite small. Therefore their main functional importance probably is to be found in their control of certain metabolic events in liver and skeletal muscle cells, which lack a direct sympathetic innervation (CELANDER 1954).

The often quite rapid decrease of heart rate on interruption of a low frequency accelerans nerve stimulation is of some interest as it probably also has a bearing on the sympathetic effect on heart contractility. If this is so, rather prompt decreases of stroke volume might be induced simply by a reflex sympathetic inhibition, especially if it includes an inhibition of a tonic sympathetic influence on the 'capacity vessels', directing the blood back to the heart. Other things being equal even a slight dilatation of this section of the vascular tree must mean a definitely decreased venous return and thus affect the filling of the heart ventricles. Effects on the heart, caused by a generalized inhibition of sympathetic tone, might thus appear just as rapidly as the reduction of the peripheral resistance which becomes evident within few seconds. There is experimental evidence of a rapid, reflex decrease of stroke volume when baroceptor fibres are activated in man (PETERSON 1950, CARLSTEN, FOLKOW, GRIMBY, HAMBERGER and THULESIUS 1956), and it has been suggested that the promptness of this effect might necessitate the assumption of a direct vagal influence on the contractility of the heart ventricles (PETERSON 1950). Judging by the often quite rapid elimination of the sympathetic effect on the heart in the present experiments, the mentioned effect on the stroke volume in man might after all be a consequence of a generalized reflex inhibition of sympathetic tone, the more so as direct vagal stimulations in man — in marked contrast to the reflex response obtained on baroceptor fibre activation — does not significantly decrease, but in fact often increases the pulse amplitude (CARLSTEN, FOLKOW and HAMBERGER 1956).

Summary.

In cats stimulation of the accelerans nerves exerts a much stronger effect on the heart rate than the hormones released at a similar activation of the adrenal medullas. This is in accordance with the characteristics of the sympatho-adrenal control of other autonomic effectors.

The heart response is sometimes seen to vanish quite rapidly on interruption of a low-frequency accelerans nerve stimulation. It is therefore discussed whether the prompt reflex decreases of stroke volume, known to occur on baroceptor stimulation in man, really necessitate the assumption of a direct vagal control of the heart ventricles. It is after all not impossible that this is the consequence of an inhibition of a tonic sympathetic influence on the heart and adjacent capacity vessels, factors which both must result in some decrease of stroke volume.

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Transcapillary Migration of Ethyl Alcohol in the Pulmonary Circulation. A Method for Determining the Water Content of the Lungs in Vivo.

By

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Analysis of indicator-dilution-curves (STEWART 1921, HAMILTON, MOORE, KINSMAN and SPURLING 1932) makes possible the determination of the amount of blood between the site of injection and the site of sampling, and consequently the determination of the blood content of separate organs.

By simultaneous injection of an easily diffusible and a non-diffusible substance, information can be obtained regarding the size of the extravascular space of the diffusible substance in a single organ.

This paper describes a method for determining the extravascular water content of the lungs in normal man measured as the ethylalcohol space of the lungs.

Methods.

When two substances were injected into a peripheral vein, one of which stayed in the vessels (*e. g.* T-1824) while the other quickly diffused through the capillary wall into the extravascular space (*e. g.* ethylalcohol), the concentrations in the arterial blood of either substance gave characteristic dilution-curves. The curve of the non-diffusible substance is determined by the size of the cardiac output and the amount of blood between site of injection and site of sampling. In addition the dilution-curve of the diffusible substance depends on the amount lost from the lung capillaries.

From the dilution-curve of the non-diffusible substance and the known concentrations of the two substances in the injecta, a curve

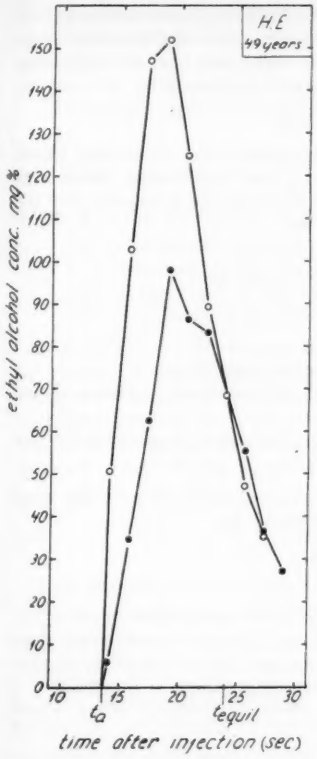

Fig. 1. ●——● observed arterial alcohol concentrations after injection of a mixture of 3 ml of T-1824 and 5 ml of 30 per cent ethylalcohol in 0.9 per cent saline. ○——○ expected arterial alcohol concentrations calculated from the arterial dye-dilution curve.

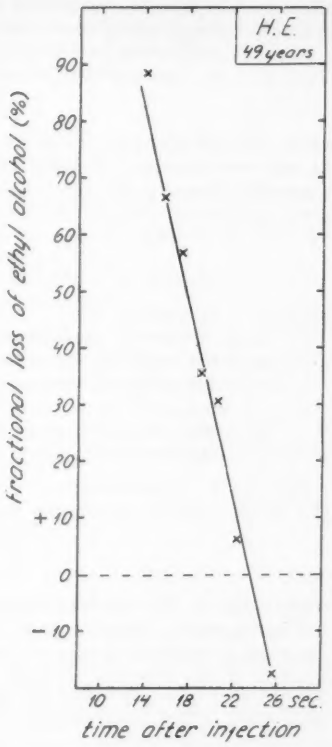

Fig. 2. The fractional alcohol loss from the lung capillaries during the first circulation. The curve is employed to determine the exact equilibrium time ($t_{equil.}$) *i. e.* the time of equilibrium between intra- and extravascular alcohol (the fractional loss = 0).

could be constructed representing the expected concentrations of the diffusible substance had no loss taken place from the capillaries.

When the observed and expected dilution-curves of the diffusible substance were drawn in the same coordinate-system, curves were obtained of a type as shown in fig. 1.

At first the observed values were lower than the expected, as a consequence of the loss from the lung capillaries during this period. This diffusion loss gradually decreased until equilibrium was obtained. From these curves it was possible to calculate the amount of diffusible

substance lost from the capillaries: if the lung capillaries are conceived as a tube through which the blood flows at a rate of f liter/min. with a constant concentration of diffusible substance in the inflowing blood (c_e), the amount lost per minute is expressed by

$$f \cdot (c_e - c_o)$$

where c_o is the concentration of the substance in the outflowing blood. As the concentrations in both inflowing and outflowing blood were constantly changing, the total amount of diffusible substance lost (Q) until equilibrium occurred was given by

$$Q = \frac{F}{60} \cdot \int_{t_a}^{t_{equil.}} (c_e - c_o)\ dt$$

where t_a = appearance time,
$t_{equil.}$ = time of equilibrium,
c_e = the expected concentration in mg/100 ml,
c_o = the observed concentration at any time between t_a and $t_{equil.}$,
F = the pulmonary water flow (the water fraction of the cardiac output) in l/min.

The space of distribution of the diffusible substance in the lungs (V) at the time of equilibrium was defined by

$$V = \frac{Q}{c_{equil.}}$$

where $c_{equil.}$ is the alcohol-concentration at equilibrium time.

The expected alcohol concentrations (c_e) were calculated from the alcohol concentration in the injecta and the measured concentrations of Evans blue dye (T-1824) (assuming complete penetration of alcohol into the red cells) by the following formula (after FREIS, HIGGINS and MOROWITZ 1953):

$$c_e = \frac{c_a^i \cdot c_{T\text{-}1824}^m}{c_{T\text{-}1824}^i + \left[\frac{h\,Wc}{p\,Wp}\,(c_{T\text{-}1824}^i - c_{T\text{-}1824}^m)\right]}$$

where c_a^i = the alcohol concentration in the injecta,
$c_{T\text{-}1824}^m$ = the measured concentration of T-1824,
$c_{T\text{-}1824}^i$ = the concentration of T-1824 in the injecta,
p = the plasma fraction of the blood,
h = the cell fraction of the blood,
W_c = the water fraction of the red cells,
W_p = the water fraction of the plasma.

The area between the two curves (fig. 1) was calculated from the appearance time to the time of equilibrium by means of the trapezoid rule. The determination of equilibrium time from the curves was difficult as the down-strokes often took a parallel course. When the fractional loss of alcohol was plotted against time, a rectilinear curve was obtained, representing a decreasing positive loss during the first

few seconds and a negative loss in the last period (fig. 2). The equilibrium time was the time at which the loss was nil. The equilibrium concentration was the mean of the expected and the observed concentrations of alcohol at the equilibrium time.

The cardiac output was given by

$$C.O. = \frac{I \cdot 60}{\bar{C} \cdot PT} \cdot \frac{100}{100\text{-}h}$$

where I = the amount of T-1824 injected in mg,
$\bar{C}$ = the mean concentration in mg/liter during the first circulation of T-1824,
PT = the time of the first circulation in seconds,
h = the hematocrit value of the blood.

$\bar{C}$ and PT were determined as described by HAMILTON et al. (1932) by plotting the measured concentrations of T-1824 on semilogarithmic paper and eliminating the effect of the recirculation by drawing the first rectilinear part of the down-stroke down to the base line.

The water-fraction of the cardiac output (F) was calculated as 90 per cent of the plasma output plus 65 per cent of the red cell output.

Material.

The investigation comprised 15 persons aged 15—50 years without pulmonary or cardiovascular diseases. In addition three experiments were made on the same normal person. The experiments were performed in the morning, the subjects being in the fasting condition or having only had a light fat-free breakfast. Before the experiments the subjects rested in a quiet room for at least half an hour (a few receiving light sedatives) to ensure basal conditions.

In local anesthesia a needle with stiletto was placed in the brachial artery. A second needle was placed in an antecubital vein of the opposite arm (usually the basilic vein). A syringe was mounted on the latter needle containing exactly weighed amounts of Evans blue dye, T-1824 (usually 3 ml of a .5 per cent solution), and ethylalcohol (5 ml of a 30 per cent solution in .9 per cent saline). A 15 cm polyethylene tube with an internal diameter of 1.5 mm was attached to the arterial needle. This tube was connected to a rotating blood-sample collecting device as designed by ASMUSSEN and NIELSEN (1952). The device made it possible to sample blood continuously in small plastic tubes at known intervals. The tubes contained a small amount of dry heparin.

Table 1.

The extravascular ethylalcohol space of the lungs in 15 normal subjects.

subject	$c_{equil.}$	$t_{equil.}$	cardiac index	extravascular alcohol space
	mg% ethylalcohol	sec.	l/min/m²	ml
H. L.	66	26	3.23	595
E. H.	48	30	2.81	562
B. N.	55	16	5.12	465
H. N.	42	32	3.14	596
N. H.	40	29	4.06	424
L. B.	60	20	3.64	367
M. J.	61	27	4.02	566
E. O.	42	32	2.78	473
J. K.	80	25	2.72	333
H. E.	68	24	3.40	429
K. H.	57	26	3.41	709
M. J.	92	18	3.77	570
J. L.	55	26	3.84	646
T. S.	88	26	3.20	450
U. L.	72	19	4.96	535
mean			3.61	515
S. D.			0.72	105
S. E.			0.19	27

The mixture of T-1824 and ethylalcohol was injected as fast as possible. The sampling device was started in the middle of the injection and arterial blood was continually collected during the following 25—50 seconds.

Immediately after the experiment the tubes were centrifuged and 100 μl of the supernatant plasma were analyzed for alcohol using the Conway method (Conway 1950).

The rest of the plasma was analyzed for T-1824 in a Beckman spectrophotometer, model DU, at 620 Å, using micro-cuvettes and diaphragm as designed by Lowry and Bessey (1946). For each experiment a standard curve was constructed by reading the densities of a series of known concentrations of T-1824 in the subject's own plasma.

The error of photometric T-1824 determinations is about 2 per cent. The error of the dye-dilution method for determining the cardiac output is about 10 per cent (Lagerlöf, Bucht, Werkö and Holmgren 1949). The error of the alcohol analysis

Table 2.

Three determinations of the extravascular ethylalcohol space of the lungs in the same normal subject (B. B., male aged 34 years).

date	$c_{equil.}$	$t_{equil.}$	cardiac output	extravascular alcohol space
	mg% ethylalcohol	sec.	l/min.	ml
Aug. 15	28	26	8.65	561
Aug. 30	46	30	7.01	524
Sept. 21	45	29	7.66	497
mean				527

is about 3 per cent. We have ascertained that only negligeable amounts of alcohol were lost by evaporation from the tubes as analyses of samples taken from the tubes after up to 1 hour after the experiment showed no measurable loss.

Results.

Table 1 shows the experimental results. We found a mean alcohol diffusion space in the lungs of 515 ± 27 ml ($n = 15$).

That the experiments have taken place under basal conditions is shown by the fact that the mean cardiac index was 3.61, which is in good agreement with the results of other investigations of cardiac output at rest.

The inconstant $c_{equil.}$ is partly due to alterations in the amount of alcohol in the injected mixture; but even when the same amount of alcohol was injected, different equilibrium concentrations were obtained. Equilibrium must be expected to occur later in the course of the dilution-curve when the extravascular volumes are large than when they are small, and consequently at lower concentrations. It is seen that equilibrium and the start of the recirculation were almost simultaneous. This means that a substance with a slightly slower diffusion rate than alcohol can not be used.

Table 2 gives the data from three experiments on the same normal subject at 2—3 weeks' intervals. They demonstrate a reproducibility within ± 10 % from the mean value.

Discussion.

A condition for the validity of the method is that the non-diffusible substance did not leave the vessels. Investigations concerning the elimination of T-1824 from the blood-stream (GIBSON and EVANS 1937) support the assumption that the loss of dye from the pulmonary vessels was negligeable during the experiment.

A second condition is that alcohol diffused exclusively to the extravascular space of the lungs. This condition is met as the loss in the great vessels must be relatively insignificant as compared to the diffusion from the pulmonary capillary bed, and the loss to the alveoli was quite insignificant even when complete diffusion equilibrium between lung tissue and alveolar air is assumed, as the coefficient of distribution between air and blood is .0005 (LILJESTRAND and LINDE 1930).

As our alcohol determinations have been made on plasma, we have had to correct for penetration into the red cells assuming complete equilibrium with their water fraction. We tried to estimate the rate of penetration of alcohol into the erythrocytes by measuring the time until total hemolysis, but this depends on other factors than the penetration of alcohol; probably injury to the cell membrane is a co-operating factor at high alcohol concentrations. If the alcohol penetration was not complete, as we have assumed, the extravascular space of distribution would be greater than our values indicate.

The calculations are only possible if equilibrium between intra- and extravascular alcohol is obtained before the beginning of recirculation. Two experiments have been disregarded in this report because equilibrium was not obtained.

The surprisingly short time necessary for the injected substance in the blood stream to reach equilibrium with that in the extravascular space agrees well with recent investigations showing that the transcapillary exchange of several substances takes place rapidly (CHINARD, VOSBURGH and ENNS 1955). 105 per cent of the plasma water is exchanged each minute with extravascular water (FLEXNER, COWIE and VOSBURGH 1948). The fast rate of equilibration is due to the short distance of diffusion in the lungs. KETY (1951) mentions that with D_2O 95 per cent equilibration is obtained in .72 seconds in a tissue with an intercapillary distance of 30 mμ. In lung tissue the mean

distance between the capillaries is of this order or less. Our experiments seem to show that ethylalcohol does not differ much from D_2O in its rate of diffusion, as complete equiblibrium between blood and lung-tissue was obtained within 20—30 seconds.

Investigations by HEVESY and JACOBSEN (1940) indicate that not only the transcapillary exchange but also the distribution through the extravascular space takes place very rapidly. It was found that injected D_2O equilibrated with the total extracellular space of rabbits in 30 seconds.

Our results indicate that alcohol was distributed to the total extravascular space during a single circulation through the lungs. The mean extravascular alcohol space in the lungs of about 500 ml can not represent the extracellular space alone if the usual ratio between intra- and extracellular space is valid in the lungs. LILIENFIELD, FREIS, PARTENOPE and MOROWITZ (1955) using a technique corresponding to our's found a D_2O-space in the lungs of about 200 ml (range: 105—360 ml). Possibly this value represents the extracellular water.

If our figures represent the total extravascular water content of the lungs, then the lung tissue weighs about 600 grams. Consequently the amount of blood remaining in the minor lung vessels and capillaries after removal from the body should be 300—400 ml. Investigations concerning "central volume" (the blood content of the large vessels and capillaries of the lungs in vivo) show a value of about 1,000 ml with a wide range (LAGERLÖF, BUCHT, WERKÖ and HOLMGREN 1949, KATTUS, RIVIN, COHEN and SOFIO 1955). The greater part of this volume must be located in the large lung vessels, the blood content of which is lost when the lungs are removed from the body.

Summary.

A method is described for determining the extravascular water content of the lungs in living humans. The method is based on a determination of the amount of ethylalcohol lost from the capillary bed during the first circulation through the lungs. When the alcohol concentration of the extravascular water was also known, the space of distribution of alcohol could be calculated. There is reason to believe that the extravascular alcohol space represents the total extravascular water content of the lungs.

In 15 normal subjects the method showed a mean value of 515 ± 27 ml (S. E., n =15). Three experiments performed on the same subject showed a maximum deviation from the mean value of ± 10 per cent.

The present investigation indicates that the total weight of the extravascular lung tissue is about 600 grams, and consequently the amount of blood in the capillaries and minor vessels of the lungs which is not lost at removal must be 300—400 ml.

The authors are indepted to Doctor Claus Brun for valuable suggestions and criticism in the course of this study.

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The Relative Rates of Formation of Acetic, Propionic and Butyric Acid in the Rumen of Sheep.

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Studies on variations in ketone bodies in ruminant blood which have been carried out at this institute, have made us interested in the quantitative aspects of volatile fatty acid formation in the rumen.

SCHULTZ and SMITH (1951) have shown that in contrast to acetic acid, butyric acid markedly increases blood ketones in goats when introduced continuously into the rumen. The *in vitro* experiments of PENNINGTON (1952 and 1954) demonstrate that the transformation butyrate to acetoacetate may take place with great rapidity in the rumen wall. This will explain the observation by KIDDLE, MARSHALL and PHILLIPSON (1951) that the ratio butyric/acetic acid in the blood leaving the rumen is much smaller than in the rumen contents.

Already in 1945 DANIELLI, HITCHCOCK, MARSHALL and PHILLIPSON showed that the ratio butyric/acetic acid for quantities disappearing from the isolated rumen of anesthetized sheep was considerably higher than the ratio between the actual concentrations in the rumen when pH was 5.8. However, at a pH of 7.5 the disappearance rates seemed to be about proportional to the concentrations. In this connection reference should be made to the extensive investigations on rumen acidity carried

out by PHILLIPSON (1942). Values for rumen pH approaching those of blood were observed only after fasting periods of 48 hours duration. All types of feeding tried resulted in pH-values on the acid side of neutrality.

The confirmation of the results of DANIELLI et al. by PFANDER and PHILLIPSON (1953) is important because in contrast to the earlier investigators they kept the acid concentrations constant and at normal levels while the disappearance rates were measured.

As will be known, a surprising constancy exists in the relative concentrations of individual volatible fatty acids in a normal rumen during fermentation (ELSDEN 1945/46, PHILLIPSON 1947, GRAY and PILGRIM 1951, TYZNIC and ALLEN 1951, GRAY, PILGRIM, RODDA and WELLER, 1952). The results referred to above seem to imply that the invariably low relative concentrations of butyric acid found, result from peculiarities in the mechanisms of absorption through the rumen epithelium. One would expect, however, that confirmatory evidence as to the importance of butyric acid should be obtainable through direct observations on fermentation rates of acids in rumen contents.

When GRAY, PILGRIM and WELLER (1951) came to the conclusion that propionic acid is quantitatively the most important product of carbohydrate fermentation in ruminants, it may have been due to the long fermentation periods used in their *in vitro* experiments. It might be difficult to secure the constancy of a mixed microflora *in vitro* with fermentation products accumulating for 48 hours.

Our aim has been to try and find approximations to fermentation rates in the rumen by measuring increases in volatile fatty acids in isolated samples of rumen contents incubated for short periods. One hour incubation periods were found practicable. The experiments reported here were carried out in the summer of 1954 and in the winter of 1954/55. In the meantime, we became aware that the short-time incubation principle had already been made use of by CARROLL and HUNGATE (1954). The main differences between their and our experiments have been that they have made their observations on steers while we have used sheep, and that their separation of volatile acids has been made according to a modified ELSDEN technique, while we have used the gas-liquid chromatography method of JAMES and MARTIN (1952). From our experiments we are able to report a somewhat higher relative rate of butyric acid formation than CARROLL and

HUNGATE. As a control we have measured the changes which took place in fatty acid concentrations within the rumen during the incubation interval.

Experimental.

All the results presented in this paper are obtained on one animal, a ewe which was one year old at the beginning of the experiments. It was provided with a permanent rumen fistula, through which was inserted an ebonite screw-cap canula of the type invented by PHILLIPSON and INNES (1939). The animal remained in good health throughout the 9 month period during which the studies were made. It was kept indoors all the time and fed on a diet of hay and mixed concentrates in quantities sufficient for weight maintenance. The daily consumption was 0.4—1.2 kg of hay and 0—0.3 kg of concentrate mixture.

Sampling. Rumen contents were obtained by suction through a glass tube of 9 mm inner diameter. The samples provided in this manner contained plenty of coarse material, and one would not expect serious errors to arise from particle fractionation. It may be difficult, however, to state with certainty to what extent an individual sample is representative for the whole rumen. Therefore all conclusions arrived at are based on average results from series of measurements on different samples covering the whole period of time between morning and afternoon feeding.

For each determination of fermentation rates two samples were drawn from the rumen. From part of the first one, liquor was separated by filtration through gauze immediately after drawing. The rest was transferred to an incubation vessel where it remained for exactly one hour before filtering. Sample number two was taken out just at the end of the hour, the fresh sample and the one from the incubator then being filtered simultaneously. All three fractions of liquor were submitted to volatile fatty acid analysis as soon as they were prepared, further fermentation being prevented by the acid reagents added at the beginning of the analyses.

The differences in concentrations of acetic, propionic and butyric acids between the two fractions from sample no. 1 were taken as measures of the rates of production of these compounds in the rumen.

The values of volatile fatty acids found in sample no. 2 served as controls. They always proved lower than those for the incubated portion of the first sample. If this had not been so, it might have meant that *in vitro* the fermentation was proceeding more slowly than inside the rumen.

The animal was never allowed to eat or drink during the period between the two samples. The extent of the dilution of ruminal contents by saliva during this period was, however, unknown. Nor did we know the quantities of fluid passing from the rumen to the omasum. Therefore it is not permisible to interpret the positive differences be-

tween incubated sample and sample no. 2 as measures of absorption from the rumen.

Incubation procedure. As a thermostat a Warburg water bath kept at a temperature of 39° C was used. Rubber stoppered Erlenmeyer flasks of 50 ml volume served as fermentation vessels. Through the stopper two narrow-bore metal canulas of the type used for taking blood samples were inserted. The flasks were preheated to the thermostat temperature before introducing the samples. During the first minute in the thermostat, a gentle stream of nitrogen (O_2 content not exceeding 0.3 per cent) was led through the flasks to wash out the air. Nitrogen was not allowed to bubble through the liquid phase. Afterwards one of the canulas was closed, the other one being left open to allow fermentation gasses to escape. Because of the outward flow of these gasses there should be little chance of air diffusing back into the flasks. Mechanical shaking was applied continuously for the duration of the incubation period.

Measurements of pH before and after incubation never revealed changes greater than 0.3 units. The one hour increase in volatile fatty acids did not exceed the magnitude of 10 per cent. It seems rather unlikely that the small accumulation of fermentation products which these figures indicate, should seriously influence microbial activities.

Analysis of volatile fatty acids. Rumen liquor was separated by filtration through gauze. A protein precipitation was performed with $CdSO_4$ and NaOH according to the procedure of FUJITA and IWATAKE for blood (1931). In the filtrate total volatile fatty acids were determined by steam distillation. All samples were analysed in this way in order to check the chromatographic results. Very close to 100 per cent recovery was found for volatile fatty acids added to liquor samples before precipitation.

The JAMES and MARTIN chromatographic technique (1952) requires the acids to be transferred quantitatively to a water-free medium of ethyl ether before they are brought into the analytical column. It adds to the difficulties that the amount of ether which can be taken up by the column together with the volatile acids is strongly limited. It was found practicable, however, to concentrate the solutions without acid loss by distilling off ether through long Vigreux reflux columns. This made possible the use of liberal amounts of the solvent. Considerable simplification was thereby obtained because extractions could be performed directly from liquor samples without previous precipitation of proteins. The amounts of water that had to be dealt with, were in this way reduced to a minimum. As the extractions were begun immediately after preparation of the liquor, no precautions had to be taken to stop fermentation.

Described step by step the analytical routine adapted was as follows. A 0.2 ml sample of the freshly separated rumen liquor was pipetted into a glass stoppered cylinder (15 ml volume) together with 10 ml newly distilled dried ether, and 0.1 ml 60 per cent H_3PO_4. After shaking for three minutes, one gram of anhydrous Na_2SO_4 was in-

troduced as a desiccant. Then followed another three minute period of shaking. Left standing, the contents of the cylinder rapidly settled and the ether could be removed by decantation. To complete the extraction, two more 10 ml volumes of ether were applied. The extracts, 30 ml altogether, were collected in a tube-shaped vessel (2.4 by 13 cm) that fitted by ground glass joint to a Vigreux column. This was 90 cm long and made from glass tubing of 7 mm inner diameter. Heating with water of about 35° C, the volume was reduced to 5 ml in this apparatus in about an hour. As an insurance against losses through the joint, a negative pressure of 40—50 mm Hg relative to that of the atmosphere was maintained within the apparatus during the distillation.

In the subsequent chromatographic separation of the acids extracted by the ether, the technique of JAMES and MARTIN was followed very closely. The columns used were 1 m long, the interior diameter being 6 mm. When the volume of ether that had to be taken into the column in one analysis was 5 ml, it was found necessary to make them as wide as this. The originators of the method state that no marked fall in the efficiency of the columns takes place with repeated use. However, with the relatively large volumes of ether in our analyses a gradual decline in separating efficiency was noticeable. Bringing the 5 ml of extract into a column by evaporation took about 45 minutes. The chromatograms obtained indicated some spreading and migration of the acid zones on the columns during this operation. Since the extractions were made directly from rumen liquor, a residue of non-volatile substance would remain in the glasses after the evaporization was finished.

The yield of the chromatographic analyses in acetic, propionic and butyric acids combined was, on the average, 91.7 per cent of the total content of volatile fatty acids as determined by the steam distillation method.

MCCLYMONT (1951) and EL SHAZLY (1952) have found acids with higher molecular weight than butyric, to constitute 3 to 5 per cent of the total amount of volatile fatty acids present in rumen contents (calculated on a molar basis). Small amounts of higher acids were also detectable on our chromatograms, but as they were difficult to measure accurately they were not included in the calculations. However, accepting the figures of the authors mentioned, our average recovery for the three acids in question should be between 94.5 and 96.5 per cent.

Results.

1. *The concentrations of individual volatile fatty acids in rumen liquor.*

Table 1 gives average values for 24 experiments performed on different days, samples being taken out 0 to 8 hours after feeding. Observations are thus included representing both periods

Table 1.

Concentrations of individual volatile fatty acids in liquor from fresh rumen samples and from samples incubated for one hour. Average values from 24 experiments.

	Acetic acid		Propionic acid		Butyric acid		Total	
	M. eqv. per l.	per cent	M. eqv. per l.	per cent	M. eqv. per l.	per cent	M. eqv. per l.	per cent
Sample I. 1. Analysed directly.	70.48	65.51	23.00	21.38	14.11	13.11	107.59	100
Sample I. 2. Analysed after one hour of incubation	75.84	64.87	25.04	21.42	16.03	13.71	116.91	100
Sample II. Taken one hour after sample I. Analysed directly	68.74	66.15	21.76	20.94	13.41	12.91	103.91	100

of increase and decrease in total acid concentration in liquor. It appears that the figures correspond very well with those cited by ELSDEN, HITCHCOCK, MARSHALL and PHILLIPSON (1945/46) and PHILLIPSON (1947) as being characteristic of fermentation in the alimentary tract of herbivora in general. They also agree satisfactorily with those reported later by CARROLL and HUNGATE for total rumen contents of bovines. As it is obvious that the concentrations result from a balance between rates of production and disappearance, they cannot be taken as direct evidence of the importance of the different acids to the animal.

2. *The production of volatile fatty acids in rumen contents in vitro.*

From table 1 it appears that the magnitudes of the increments for the three acids in milliequivalents per liter during one hour of incubation will place them in the following order of importance: acetic > propionic > butyric. The increments are, however, not proportional to the initial concentrations. After incubation the volatile fatty acid mixture from the liquor is slightly richer in butyric acid than before. The change has taken place at the expence of acetic acid.

On the average the incubation has led to an increase of 8.66 per cent in the total concentration of all three acids taken to-

Table 2.

Fermentation of rumen contents in vitro. Average results from 24 experiments.

Volatile fatty acids produced per liter of rumen liquor during one hour of incubation at 39° C.

Units		Acetic acid	Propionic acid	Butyric acid	Total
Millieqv./l.	Absolute figures	5.36	2.04	1.92	9.32
	Per cent of total	57.46	21.91	20.63	100
Gram/l.	Absolute figures	0.322	0.151	0.169	0.642
	Per cent of total	50.15	23.52	26.33	100
Kg-cal./l.	Absolute figures	1.122	0.749	1.007	2.878
	Per cent of total	39.00	26.03	34.97	100

gether. Table 2 presents calculations of the increments for each acid measured in m.eqv./l, g/l and kg-cal/l. Corresponding relative values are given as per cent of the total increment.

The figures show the amount and composition of the acid mixture one would have to add to one liter of liquor from fresh rumen contents in order to obtain concentrations equal to those found after one hour of incubation. The calory equivalents are calculated assuming the following values for heat of combustion in kg calories per g molecule, acetic : 209.4, propionic : 367.2, butyric acid : 524.3.

Discussion.

Table 1 shows butyric acid to be present in liquor in concentrations representing only about $^1/_3$ of the total for all three acids when comparisons are made on a molar basis. From table 2 it is seen that butyric acid constitutes about $^1/_5$ of the total amount produced in one hour if the same units of measurement are applied. However, in terms of calory content of the acids formed, butyric acid will represent somewhat more than $^1/_3$ of the total. This would give the following order of biological importance as sources of energy: acetic > butyric > proionic acid. Butyric acid ranges definitely above propionic and is not very much inferior to acetic acid.

Table 3.

Comparison of volatile fatty acid mixture in fresh rumen liquor with increment observed during one hour of incubation.

Average composition and standard error in moles per cent, 24 experiments.

	Acetic acid per cent	Propionic acid per cent	Butyric acid per cent
Rumen liquor	64.77 ± 0.68	22.50 ± 0.80	12.73 ± 0.32
One hour incubation increment in liquor	55.95 ± 3.60	20.61 ± 2.06	23.44 ± 2.78
P-value on difference	$0.05 > P > 0.01$	$P > 0.2$	$P < 0.001$

Table 3 contains the results of a statistical analysis comprising the results from all the experiments performed. The P-values are calculated according to the Student's t-test. There will be some discrepancy between the average figures arrived at here and those given in tables 1 and 2. This is due to different ways of calculating. The first two tables give percentages of means while the figures in table 3 are means of percentages.

The difference in composition between volatile acid mixture extractable from fresh rumen liquor and the calculated increment during one hour of incubation, has been found highly significant for butyric acid. There is also a significant difference for acetic acid. For propionic acid the ranges of variation overlap to such an extent that the difference becomes entirely insignificant. From the figures it appears that the higher proportion of butyric acid in the increment is connected with a lowering of the percentage of acetic acid. When, however, a higher P-value has been found for the latter acid than for the former, it is due to the fact that in some incubations the increase in butyric acid took place at the expence of propionic acid instead.

The standard errors of the mean for the increments are much greater than for the determinations of total amounts present. This is inevitable since the increments are of the order of magnitude of only 8 to 9 per cent of the actual concentrations, and are arrived at indirectly by subtraction. It must, however, be taken into account that the measurements are made at differing hours after feeding and that the feed rations have not been kept strictly constant. It is not unlikely that this has lead to greater variations in the relative rates of formation than in the relative concentrations.

The number of observations is not large enough to permit subdivisions of the material to be made for the calculation of the effects of changes in the fermentation substrate of the rumen. Further information on the influence of substrate composition on fermentation rates will, however, be sought in experiments which are planned for the future.

The present results support the findings of PFANDER and PHILLIPSON on rates of disappearance of individual volatile fatty acids from the rumen of sheep, mentioned in the introduction to this paper. They showed that when butyric acid is found in lower concentrations in the rumen than acetic and propionic, it may be because it is more efficiently drained out through the rumen wall than the other two are. Our conclusion is that the quotient: *rate of acid formation/acid present* has been found greater for butyric than for acetic and propionic acid. This is the outcome one would necessarily arrive at if the absorption mechanisms of the rumen of our animal were working as indicated in the experiments referred to. In this connection it is noteworthy that a highly significant tendency has been obtained in spite of substrate variations due both to changes in feeding and differences in stage of feed decomposition in the rumen at the time of measurement.

The presence of the same tendency in the results published by CARROLL and HUNGATE, who incubated rumen contents from bovines for 1 to 4 hours, also points to the influence of common mechanisms of rumen absorption. The tendency is, however, not so distinct in their material as in ours. According to their own statement, the main purpose of their studies was to provide support for the hypothesis that a major part of the bovine energy requirement is met by the volatile acids produced in the rumen. Besides, they wanted to demonstrate the potentialities of the short-time incubation method. They are themselves aware that with the small number of observations they have made on rates of formation of individual acids, they reach only a low degree of statistical accuracy. Thus the differences obtained between hay-fed, grain-fed and pasturized animals may be entirely incidental. Some of their samples are obtained by rumen fistulae, others by use of stomach tube, the latter being poorer in solid content. This may have contributed to the scattering of the results.

For the interpretation of results obtained by short-time incubation it is important that the two authors have found *in*

vitro fermentation rates for total volatile fatty acids only slowly decreasing during four hours of incubation.

In comparing the findings of CARROLL and HUNGATE with the present ones, it should be remembered that different analytical methods have been employed. The gas chromatography procedure will probably permit the determination of butyric acid with less interference from acids of higher molecular weights that might be present, than the method of ELSDEN. Otherwise, one would of course expect the quantitative results to be influenced by feed composition. Thus the calory ratios acetic: butyric for production found by CARROLL and HUNGATE (45.6 : 25.3 to 42.6 : 31.7) and the value from our experiment (39 : 35) may be equally representative. The corresponding ratio of PFANDER and PHILLIPSON based on rates of disappearance is lower (26.5 : 47.5). This does not necessarily mean that the figures are contradictory. They have investigated the ability of the rumen epithelium to absorb, while our experiments are concerned with the quantities actually offered for absorption under normal conditions.

The results reported in the present paper should be considered as preliminary. More experiments will have to be done on the connection between feed composition and the rates of formation of volatile acids. The importance of the sampling technique for the *in vitro* results should be carefully examined. The desirability of simultaneous determinations of rates of production and rates of disappearance and absorption is evident.

Summary.

1. Using the method of gas-liquid partition chromatography of JAMES and MARTIN the concentrations of acetic, propionic and butyric acid have been measured in rumen liquor from a sheep fed hay and concentrates. The results are within the ranges considered to be characteristic of ruminant digestion. Average values from 24 experiments were: total concentration about 100 milliequivalents per l, relative concentrations in moles per cent acetic: propionic: butyric = 65.5 : 21.4 : 13.1.

2. Repetition of the liquor analyses after incubation of the unseparated rumen contents for one hour at 39° C under nitrogen atmosphere, showed an increase in volatile acid content of 8.66 per cent. The average composition found for the increment in

moles per cent was acetic : propionic : butyric = 57.45 : 21.9 : 20.65. This indicates that the animal studied has derived almost as much metabolic energy from butyric as from acetic acid.

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